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MODERN CONCEPTS OF THYROID PHYSIOLOGY

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MODERN CONCEPTS OF THYROID PHYSIOLOGY*

Consulting Editor and Conference Chairman

RULON W. RAWSON

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INTRODUCTORY REMARKS

Rulon W. Rawson

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Nearly one and one-half years ago, George Mangun of the Warner-Lambert Laboratories, Morris Plains, N. J., invited me on behalf of The New York Academy of Sciences to organize a program on the physiological, pharmacological, and possible therapeutic effects of thyroxine and its various analogues. I accepted this invitation with enthusiasm, but with the provision that the theme of the conference on which this monograph is based should be *Modern Concepts of Thyroid Physiology*. I also obtained the understanding that I should have the assistance, as vice-chairman, of my colleagues J. E. Rall of the National Institutes of Health, Public Health Service, Bethesda, Md., and Robert Kroc of the Warner-Lambert Research Institute.

It has been eleven years since the Academy published *Thyroid Function as Disclosed by Newer Methods of Study*,¹ its last monograph on the thyroid. That publication was planned by J. H. Means and included as formal contributors E. D. Goldsmith, E. de Robertis, Edward W. Dempsey, W. T. Salter, I. L. Chaikoff, Alvin Taurog, D. A. McGinty, E. B. Astwood, C. P. Leblond, E. P. Reineke, A. Albert, and myself. It was a most stimulating discussion of the functions of the thyroid gland as determined with the tools of study then available. The monograph has been known among thyroidologists for many years as the little brown bible of thyroidology.

Since January, 1949, when that publication appeared, research in the field of thyroidology has expanded at an unprecedented pace. The availability of many and varied new tools of study developed in the laboratories of physicists, physical chemists, and organic chemists has made it possible to approach many previously unanswerable problems with workable methods. Studies with radioactive iodine have made it possible to investigate thyroid physiology at levels previously unattainable. The various chromatographic techniques combined with isotopic and other physical chemical methods have made it possible to isolate previously unidentified iodinated compounds in the thyroid and in the serum, and to study the methods by which they are transported. With such methods of separation and identification it has been possible to study the metabolism of the thyroid hormones by different tissues in various metabolic states.

I certainly do not suggest that all of the advances that have been made during the past twenty or more years have been due only to the technical tools that we have available. It is obvious to all of us engaged in research that the most important reasons for this rapid and continued growth in our knowledge of the thyroid is the fact that knowledgeable, imaginative, and enthusiastic investigators from many and varied disciplines have been attracted to use the newer tools of investigation in studying the problems of thyroid physiology, normal and morbid. This monograph affords us an opportunity to learn of new and exciting studies in thyroid physiology carried on by zoologists, physicists, organic chemists, biochemists, immunologists, enzyme chemists, physiologists,

cellular physiologists, pharmacologists, iatrochemists, physicians, and psychiatrists.

At this time it would be quite proper and appropriate to review the progress made in the past eleven years by contrasting the concepts presented in this publication with those presented in the 1949 monograph. On the other hand it might be bold, but much more entertaining, to consider in the light of the studies being presented today what directions will be followed by investigating thyroidologists during the next decade.

The recent demonstrations that several of the hormonal effects of the thyroid can be dissociated by structurally altering the thyroxine molecule suggest that the many and varied effects of the thyroid hormone are not all dependent on one action of thyroxine, but that these varied effects may be dependent upon different cellular or enzymatic reactions that may be induced by different molecular combinations.

The various but different methods of studying at the cellular level the mechanism of action of the thyroid hormone described in this publication give us reason to hope for definition and clarification of the methods and systems by which the thyroid hormones exert their actions. Systematic investigations by cellular physiologists and biochemists into the mechanism of action of thyroid hormones and of various isomers of thyroxine now available, if correlated with the various effects of these agents in experimental animals and in human myxedema, may help us to clarify the primary biochemical reactions by which these physiological reactions occur. Such correlated studies may help us to clarify the mechanisms by which the thyroid influences growth, maturation, heat production, the metabolism of body fats, and normal cerebration.

The recent demonstration that hypothyroidism in man is associated with hyperuricemia and that certain derivatives of thyroxine will increase the clearance of uric acid, whereas other derivatives do not, suggests the desirability of investigating the effects of thyroid hormone on nucleic acid metabolism.

Recent demonstrations that certain derivatives of thyroxine may profoundly influence the blood levels of cholesterol in doses that are not calorogenic suggest the desirability of studying the mechanisms by which the thyroid influences cholesterol and lipid metabolism and the possible relationships of the thyroid to the genesis of atherosclerosis.

The stimulating reports given here on the effects of certain thyroid hormones on the metabolic and clinical states of certain patients with mental illness may open entirely new areas of research, not only in the field of thyroid physiology, but also in the field of psychophysiology and psychopharmacology. These observations also suggest that we might reopen previous areas of investigation into the relationships of the thyroid hormones to the production and actions of certain catechol amines, other hormones, and metabolic products known to influence cerebral function.

The recent demonstrations of more than one large iodinated protein molecule within the thyroid, along with the demonstration of two or more iodinated thyronines in the thyroid, suggest that there may be different pathways of hormone synthesis or of proteolysis in the thyroid, and that these different pathways or breakdown systems may account for the development of certain

diseases of the thyroid. These studies, if not already under way with human material, will surely soon be undertaken.

Recent investigations on antibodies to thyroid proteins and on the effects of triiodothyronine on the production of gamma globulin suggest the desirability of investigating the effects of thyroid hormones or their isomers on immunophysiological mechanisms.

The intensive attack on the thyrotrophic hormone may soon result in a pure preparation of this hormone with which we might clarify its action and mechanisms of action. We might then possibly be able to determine what role, if any, this hormone plays in the production of certain diseases of the thyroid. Studies with chemical derivatives of thyrotrophic hormone will help us to determine the essential groups of this hormone and to clarify its mode of action. Such studies may also lead to therapeutic tools with which alterations in growth or function of the thyroid might be reversed.

If this crystal-ball gazing is more or less accurate, the chairman of the Academy's next conference on the thyroid will no doubt have a very broad field of studies, both basic and practical, from which to choose his program. On the other hand, that chairman a decade from now may be greatly amused by my naïveté.

Reference

1. ANNALS OF THE NEW YORK ACADEMY OF SCIENCES. 1949. Thyroid Function as Disclosed by Newer Methods of Study. **50**(5): 279-508.

Part I. Intrathyroidal Biochemical Reaction

IODINE METABOLISM IN LOWER VERTEBRATES

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In 1951, Lynn and Wachowski¹ wrote, as a conclusion of their survey on the thyroid gland and its function in cold-blooded vertebrates: "The use of radioactive iodine has yielded important information concerning the synthesis and release of the thyroid hormone in mammals and it promises to be equally productive in similar studies in lower vertebrates." Since that time the extensive utilization of I^{131} combined with paper chromatography has permitted a deeper knowledge of iodine metabolism in poikilotherms, and it is the purpose of this study to assemble the information recently acquired in this domain. These new techniques, however, should not lead to the rejection or neglect of past techniques, such as histology and the chemical determination of I^{127} , which retain their value. An exhaustive study of thyroid function of lower vertebrates should be conducted with the application of all available methods.² For example, the determination of the blood iodine is indispensable to an appreciation of the significance of the uptake of I^{131} by the thyroid in cold-blooded vertebrates. Indeed, the great variety of environments in which they live (terrestrial, aquatic, rich or poor in iodine) and the faculty of some species of changing from one environment to another involve great variations in the level of blood iodine.

In this report, the different phases of intra- and extrathyroidal metabolism of iodine (I^{131} and I^{127}) and its variations under the influence of different ethologic or ecologic factors will be discussed. In spite of its importance, the physiological role of thyroid hormone in lower vertebrates will not be considered, as many excellent reports already have been devoted to this problem.^{1,3,4}

Extrathyroidal Distribution of Iodide

TISSUE

In mammals, a number of tissues in addition to the thyroid, possess the property of concentrating iodide, that is, the gastric mucosa, the salivary glands, the lactating mammary gland, and the placenta.⁵ In lower vertebrates an identical property is found in other tissues, that is, the notochord in cyclostomes, the gills, stomach, and bile in selachians, the skin in Amphibia, and the ovary in cyclostomes, teleosts, and Amphibia. Since iodide concentration in the ovary has been found also in birds,^{6,7} and the ovary of mammals is rich in iodine,⁸ it thus seems to be a general phenomenon in the vertebrate series.

Cyclostomes (Table 1)

In the marine lamprey (*Petromyzon marinus*) captured during its reproductive migration, the notochord accumulates 12 per cent of the injected dose of I^{131} in the female and 25 per cent in the male.⁹ The difference between the

two sexes is explained by the fact that the testis accumulates much less than does the ovary. The ratio of concentrations, notochord:plasma, attains values greater than 100 three days after injection of I^{131} . A similar mechanism of transport of iodine against a concentration gradient also exists in the notochord of the ammocoete and the adult of the *Lampetra planeri*.¹⁰ The determination of stable iodide (I^{127}) shows a content of iodide of the notochord 100 to 300 times higher than that of the plasma. The fixed iodide is localized principally in the large vacuolized cells of the notochord and remains in the form of inorganic iodide, as indicated by its solubility and chromatographic characteristics. The concentration of iodide is inhibited by thiocyanate, but evidently not by thiourea, and is not modified by thyrotropin (TSH).^{*} It seems therefore that the mechanism of iodide concentration in the notochord is similar to that observed in different tissues of mammals. Bromine is concentrated equally by the notochord, but much less than is iodide, and the content of chlorine of the notochord is equal to that of the plasma.

TABLE 1
UPTAKE OF I^{131} BY OVARY AND NOTOCHORD OF FEMALE MARINE LAMPREY*

Body weight (gm.)	Ovary weight (gm.)	Hours after injection	Injected I^{131} recovered (%)		I^{131} /gm. organ I^{131} /gm. plasma	
			Ovary	Notochord	Ovary	Notochord
662	162	2	3.4	2	0.15	0.5
900	117.5	12	10.7	8.6	1.3	3.5
870	123	24	12.8	13.9	2.2	8.5
960	161	24	26.8	11.9	4.9	9.1
950	120	72	61.3	12.8	170	103.3
735	102	120	70.8	12.9	198	124.8
1020	152	192	52.3	11	143	112.5

* *Petromyzon marinus marinus* L.

The quantity of I^{131} taken up by the maturing ovary of the marine lamprey and of *L. planeri* is considerable¹¹ because of its great capacity for concentrating iodide and the considerable mass of the ovary (one eighth to one quarter of the weight of the body); TABLE 1. Thus the ovary can accumulate up to 70 per cent of a dose of injected I^{131} . The amount of I^{127} in the ovary of the marine lamprey is 50 to 100 times that in plasma. The major part of the I^{131} fixed in the ovary is in the inorganic form. However, the determination of I^{127} reveals the presence of certain quantities of iodide that precipitate with 10 per cent trichloroacetic acid. Synthesis of organic iodine compounds is therefore not excluded. Thiourea diminishes the concentration of I^{131} in the ovary. It is the same for TSH, which also diminishes the total quantity of I^{127} in the ovary of *L. planeri* (unpublished data).

Selachians

In the dogfish *Scyllium canicula* injected with I^{131} , the ratio of concentration, gills:blood (8 and 18, respectively), 8 and 24 hours after injection, indicates a

* Unpublished data.

possible excretion of radioiodide by the gills (unpublished data). Twenty-four hours after injection the concentration in the stomach is 39 times higher than that in blood, and is certainly higher if the gastric juice is considered. At this time the stomach contains 27 per cent of the injected dose. Therefore, the existence of a gastric "iodide pump" is observed in selachians, analogous to that described in mammals. In the case of the bile, the ratio bile: blood is 5.6 in 8 hours and 10.2 in 24 hours. It seems certainly to involve iodide concentration since, during this time, there is no hormonal iodine in the blood. Finally, the small developing ovocytes seem to concentrate iodide, but the scarcity of facts does not permit definitive conclusions.

Teleosts (Table 2)

The maturing ovary of the rainbow trout can accumulate up to 70 per cent of the I^{131} injected (unpublished data). The lesser accumulation in the ovary

TABLE 2
 I^{131} AND I^{127} CONTENT OF OVARY OF SOME TELEOSTS

Species	Body weight (gm.)	Ovary weight (gm.)	Hours after injection	Injected I^{131} recovered in ovary (%)	I^{131} /gm. ovary I^{131} /gm. plasma	I^{127} /gm. ovary I^{127} inorganic/gm. plasma
Rainbow trout (<i>Salmo gairdnerii</i>)						
Maturing	150	12.6	72	70	17.4	14.2
Ripe and spent	—	—	—	—	—	7.8*
Sea-run steelhead	—	—	—	—	—	9.4*
Salmon (<i>Salmo salar</i> L.)						
Maturing	5.000	27	24	7	1.33	—
	4.900	37	24	6.7	0.72	3.4
	4.100	22.1	72	7.75	2.00	5.5
	7.100	—	72	—	2.90	—

* Data from Robertson and Chaney.¹²

of the salmon (unpublished data) and the low concentrating ability in regard to the plasma are probably due to high blood iodine and to the greater binding of the iodides to plasma proteins (*see below*). The determination of I^{127} shows that the concentration of iodide in the salmon and trout ovaries is about 10 times that in the plasma. Ratios of the same type have been pointed out in *S. gairdnerii* and in the mature sea-run trout of California.¹²

Lungfishes

In *Protopterus annectens* no tissue seems to concentrate iodide in significant quantities. However, the radioactivity of small ovocytes is always greater than that of the plasma. The ratio ovary:plasma varies from 1.4 to 1.7 (unpublished data).

Amphibia

The concentration of iodide by the ovary of the frog *Rana temporaria* L. has been demonstrated by Volpert *et al.*¹³ The ovary can accumulate up to 43 per

cent of the injected dose and the ratio of ovary:plasma radioactivity is approximately 1 at 24 hours after the injection and attains an average of 4 at 2 to 6 days after the injection. Similarly, we have observed a concentration of iodide in the ovary of *R. esculenta* (unpublished data). According to Volpert *et al.*, the iodide that is fixed remains in the inorganic form.

The fixation of iodide by frog skin has been observed in *R. pipiens*¹⁴ and *R. temporaria*^{13,15} but, according to Volpert,¹³ the small ratio of skin I^{131} to plasma I^{131} does not permit the conclusion that this tissue actively concentrates iodide except for localization of these processes in certain cellular elements. According to Gennaro and Clements,¹⁴ the largest quantity of isotope accumulates in the dark dorsal skin rather than in the light ventral skin, and the iodide is incorporated in the organic compounds moniodotyrosine (MIT) and diiodotyrosine (DIT). TSH and also intermedin increase^{14,15} the uptake of I^{131} in the skin. Treatment with thiourea diminishes the degree of incorporation of I^{131} in the melanized areas,¹⁶ in agreement with the findings of Dent and Hunt,¹⁷ who demonstrated this in the tadpoles of *Hyla versicolor*, *Bufo americanus*, and *R. palustris* by autoradiography. This localization of organically bound iodine in the pigmented tissues is not observed after treatment by phenylthiourea.¹⁸ The same authors demonstrated an accumulation of organically bound iodine in the thymus of the tadpole which is completely inhibited by antithyroid drugs.

Reptiles

Shellabarger *et al.*¹⁹ have studied iodine metabolism in the turtle *Pseudomys floridanus*. The majority of the tissues examined contained little I^{131} .

BLOOD IODIDES

Distribution of Iodide in Red Blood Cells and Plasma; a Binding of Iodide with One or Several Plasma Proteins in Certain Teleosts

The distribution of blood iodide studied *in vivo* after I^{131} injection, or *in vitro* after adding a drop of I^{131} to blood, and expressed by the ratio I^{131} per gram of red blood cells (RBC)/ I^{131} per gram of plasma (H/P) varies with the species²⁰ (TABLE 3). Even though the H/P of selachians (0.45) or *Protopterus* is near the values observed in mammals and birds (0.40 to 0.60)²¹⁻²⁴ that of teleosts can be similar (eel, carp, conger, sea perch) or lower (Salmonidae, shad, mullet), the minimal value (0.098) being observed in the salmon during its upstream migration. This low H/P of the salmon indicates that the blood iodide is found exclusively in the plasma, while in the same species the chlorides largely penetrate the RBC.²⁰ This behavior of the iodides is comparable to that generally observed for thyroxine (T_4), which diffuses into RBC very little or not at all. This leads us to the hypothesis²⁵ that the factor limiting the penetration of iodides into salmon RBC was, as for T_4 , a binding of I^- ion with one or several plasma proteins. This hypothesis has been verified by three groups of experiments.²⁶

Experiments with washed red blood cells. RBC of salmon, rainbow trout, and eel were washed 3 times with isotonic salt solution, to eliminate all traces of plasma. They were suspended in a volume calculated according to the hematocrit with either an isotonic salt solution or a salt solution containing an in-

creasing proportion of plasma of the same animal. The H/P of the eel (FIGURE 1) does not vary in spite of the treatment. The salmon and the rainbow trout H/P in normal blood are 0.09 and 0.22, respectively: these ratios increase to 0.69 and 0.65 when the plasma is replaced by a salt solution, and decrease progressively as the proportion of plasma added to the salt solution increases.

Equilibrium dialysis of plasma. Two ml. of plasma were dialyzed with electromagnetic agitation at 4° C. for 48 hours in 15 ml. of isotonic salt solution in the presence of a drop of carrier-free I^{131} added to the interior or exterior of the dialysis bag. The concentration ratio, I^{131} per milliliter of plasma/ I^{131} per milliliter of dialyzate (R) is equal to or near 1 (TABLE 3) in the species where

TABLE 3
ERYTHROCYTE/PLASMA I⁻ RATIO (H/P) AND DIALYSIS RATIO (R) IN SOME FISH

Species	H/P ratio	Dialysis ratio (R)
Selachians		
Dogfish (<i>Scyllium stellare</i> Flem.)	0.43 (1)*	1.04 (1)*
Dogfish (<i>Scyllium canicula</i> L.)	0.45 (6)	1.00 (2)
Teleosts		
Eel (<i>Anguilla anguilla</i> L.)	0.47 (3)	1.01 (1)
Conger (<i>Conger conger</i> L.)	0.60 (1)	1.04 (1)
Carp (<i>Cyprinus carpio</i> L.)	0.40 (11)	1.03 (3)
Sea perch (<i>Labrax lupus</i>)	0.49 (2)	1.30 (2)
Mullet (<i>Mugil</i> sp.)	0.23 (4)	6.8 (3)
Shad (<i>Alosa alosa</i> L.) in upstream migration	0.23 (1)	10.3 (1)
Trout (<i>Salmo fario</i> L.)	0.19 (3)	11.3 (1)
Rainbow trout (<i>Salmo gairdnerii</i> Rich) (immature)	0.23 (20)	16.5 (2)
Sea trout (<i>Salmo trutta</i> L.) in upstream migration	0.12 (3)	25.0 (2)
Salmon (<i>Salmo salar</i> L.) in upstream migration	0.098 (15)	32.4 (9)
Lungfish		
<i>Protopterus annectens</i>	0.47 (3)	—

* The number of determinations made for each species is given in parentheses.

the H/P is between 0.40 and 0.60. However, it is clearly greater than 1 in the species having a low H/P and attains its maximal value, 32.4, in the salmon during upstream migration. Ingbar and Freinkel²⁷ in their experiments with equilibrium dialysis on human serum obtained a ratio equal to 1.2 and consider iodide bound to serum albumin. This binding, if it exists, is not significant.

Paper electrophoresis. Comparative electrophoresis with Veronal buffer was done on rat, rainbow trout, and salmon plasmas, to which a drop of I^{131} was added. Scanning the radioactivity along the electrophoregram before staining with amido black led to the localization (FIGURE 2) in the rat of one radioactive peak situated clearly far in front of the zone of protein migration and corresponding to free iodide. In the rainbow trout and the salmon there are two peaks of radioactivity, one corresponding to free iodide, the other situated in the zone of protein migration. This last peak is the most important for the salmon plasma, which has a binding capacity higher than that of the trout.

In certain salmons the peak corresponding to free iodide is sometimes totally absent, indicating that all the plasma iodide is bound. It is difficult to identify the different fractions of serum proteins in the fish. Nevertheless, a comparison of the electrophoregrams of the rat, salmon, and rainbow trout, made simultaneously, leads to the assumption that the iodides are bound in the albumin zone of the trout, and in the slow albumin or α_1 globulin zone in the salmon. Further studies are necessary to define precisely the characteristics of the protein(s) binding the I^- ion and the nature of this binding. However, the binding is destroyed by trichloroacetic precipitation of proteins.

From a zoological point of view, it seems that the iodide binding exists only in the teleosts* and, in this group, among the migratory amphibiotic species,

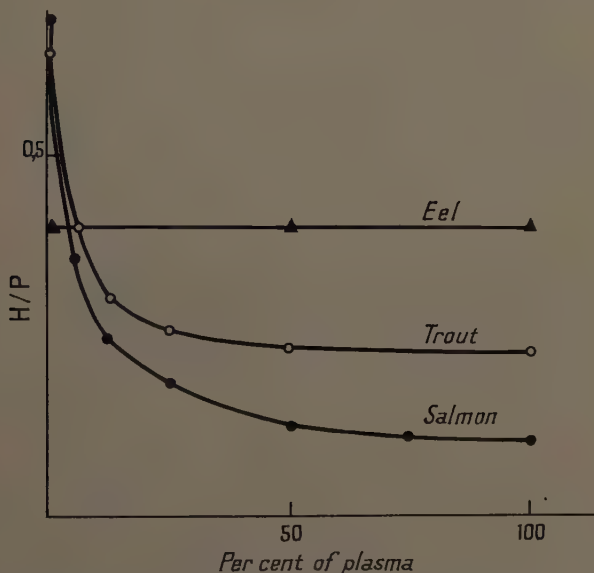


FIGURE 1. Variations of the H/P ratio in eel, salmon, and trout in relation to the proportion of plasma contained in the salt solution added to erythrocytes.

salmon, shad, sea trout, rainbow trout, and mullet. It may also appear in a genus containing migratory species.

The variations of binding capacity have been studied in the salmon (*S. salar*) at different steps of its life cycle by simultaneously measuring the H/P and the dialysis ratio (TABLE 4). The binding decreases in the sexually mature animal, whatever the sex, and it is greater during migration than in sedentary conditions. Actually, the young male sedentary parr, captured in winter on the spawning grounds, then sexually mature, has an H/P of 0.33 and an R of 5.9. However, the parr captured in the spring, whose testes have regressed has an H/P of 0.19 and an R of 13.9. The adult migrating salmon in the process of maturation has an H/P of 0.098 and an R of 32.4, while the mature salmon has

* The H/P of the marine lamprey has not been measured but, as judged by the ratio, I^{131} per gram of blood/ I^{131} per gram of plasma, an iodide binding does not seem to exist in this species.

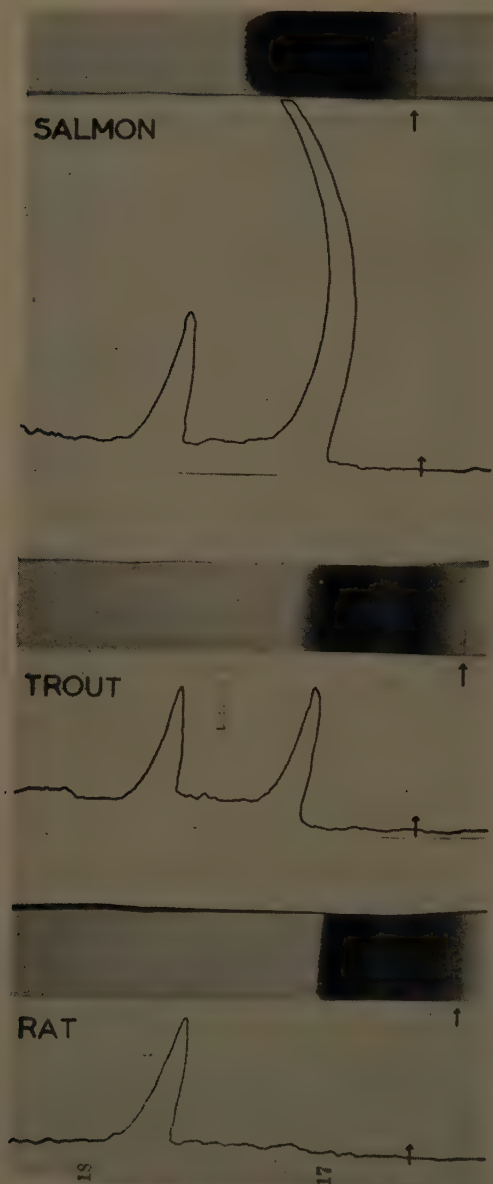


FIGURE 2. Zone electrophoresis of plasma + I^{131} of rat, trout, and salmon (barbital buffer, $\Gamma/2 = 0.1$). These plasma were run simultaneously on the electrophoresis apparatus. The paper strips, stained with amido black, are aligned with records of their radioactivity. The arrows indicate the points at which the plasma was applied.

an H/P of 0.20 and an R of 13.1. Finally, the binding increases in the migratory smolt in relationship to the sedentary states of the salmon: the parr and the parr that has become silver-coated but has been prevented from migrating. A decrease in iodide binding is similarly observed in sexually mature *S. gairdnerii*.

Plasma Iodide (I^{127})

The plasma iodide (total iodine minus protein-bound iodine [PBI]) of lower vertebrates, evaluated by the catalytic method,^{28,29} has been determined only in cyclostomes, selachians, teleosts, and lungfish. It is extremely variable, (TABLE 5). However, some general conclusions may be reached. The plasma iodide of lamprey and lungfish is low and of the same order as in mam-

TABLE 4

VARIATION OF ERYTHROCYTE/PLASMA I^- RATIO (H/P) AND DIALYSIS RATIO (R) IN SALMON AND RAINBOW TROUT IN RELATION TO MIGRATION AND SEXUAL MATURITY

Stage	Month	State of gonads	H/P ratio	Dialysis ratio (R)
Salmon (<i>Salmo salar</i> L.)				
Parr ♂	December	Ripe	0.33 (8)*	5.9 (2)*
Parr	March	Immature	0.19 (3)	13.9 (2)
Silvered parr	March	Immature	0.255 (2)	11.1 (1)
Smolt migrating	March	Immature	0.16 (3)	19.3 (2)
Adult in upstream migration	March	Maturing	0.098 (15)	32.4 (9)
Adult (at spawning)	December	Ripe	0.20 (3)	13.2 (6)
Mended	March	Spent	0.216 (11)	13.3 (10)
Rainbow trout (<i>Salmo gairdnerii</i> Rich)				
Adult ♂ + ♀	November	Immature	0.23 (16)	16.5 (2)
	April			
Adult ♀	November	Maturing	0.31 (1)	—
Adult ♀	December	Ripe	0.42 (1)	3.1 (1)

* The number of determinations for each stage is given in parentheses. In the case of young salmon, the ratios are measured on blood collected from several animals.

mals and birds. That of marine teleosts is greater, in general, than that of fresh-water teleosts because of the elevated iodine content of the environment. The highest level of plasma iodide is observed in the amphibiotic migratory teleosts captured in fresh water at the beginning of the upstream migration.^{30,31} This fact is particularly interesting when it is considered that the migrators are caught in a medium poor in iodine and that they do not eat. It would appear, therefore, that these species have a great capacity for retaining iodine. It is probable that the binding of iodides, which attains its maximal value here, is a very important factor in maintaining a high level of iodine. This permits synthesis of the thyroid hormone that is necessary for the important metabolic demands imposed by upstream migration.

EXCRETION OF IODIDES

In mammals the major route of excretion of iodide is through the kidney.⁵ In fish the kidney often plays a small role in excretion while the gills may play

an important one. However, since no comparative study of the renal or extrarenal excretion of iodides has yet been made, we shall consider the total excretion in this report.

Cyclostomes

In marine lampreys, during reproductive migration, the excretion of I^{131} is slow and small; for example, 5 days after the injection 15 per cent of the dose has been excreted. This small excretion is explained by the affinity of the notochord and ovary for iodides.¹¹

TABLE 5
PLASMA IODINE IN CYCLOSTOMES AND FISH

Order or species	$\mu\text{g. } I^{127}/100 \text{ gm. plasma}$			
	Total		PBI	
	Min.	Max.	Min.	Max.
Cyclostomes				
Lamprey (<i>Petromyzon marinus</i>)*, ³²	5.4	14.8	2.4	10.1
Selachians				
Torpedo (<i>Torpedo marmorata</i>) ³³	2.7	24	2.4	10.8
Dogfish (<i>Scyllium canicula</i>)†	8.2	51	2.4	12.5
Teleosts				
Fresh-water ^{12, 31, 34}	2.7	46	1	15
Marine ³⁵	15	91.5	6	35
Amphibiotic on upstream migration				
Salmon ^{31, 34, 36}	36	257	10	80
Sea trout ^{31, 34}	260	576	54	109
Shad ^{30, 31, 34}	248	2300	17.2	70
Lungfish				
<i>Protopterus annectens</i> ³⁷	4.2	6.4	1	3.2

* In part, unpublished data.

† Unpublished data.

Selachians

The excretion of I^{131} is extremely rapid in *S. canicula*: 57 to 72 per cent of the injected dose in 24 hours, and 94 per cent in 3 days. This rapidity of elimination of iodides is related to the concentration of I^{131} in the gills. These organs may play a preponderant role in iodide excretion. Smith³⁸ has noted an excretion of chloride by the gills of selachians.

Treatment with antithyroid drugs slows the iodide excretion. However, the blood radioiodine disappears rapidly. This apparent contradiction is explained by the iodine retention in the tissues, notably in muscle. Thus the ratio of I^{131} per gram of muscle/ I^{131} per gram of blood, which is 0.25 in the normal animal, attains 11.8 in the treated animal.³⁹

Teleosts

Marine teleosts. In the mullet *Mugil auratus* 25 and 45 per cent of the dose are excreted in 24 hours and 72 hours, respectively. On the other hand, in the

Conger conger the excretion seems much slower. In the two species treatment with antithyroid compounds strongly accelerates the elimination⁴⁰ of iodide, in accordance with the facts obtained in mammals.⁴¹

Fresh-water teleosts (Table 6). The excretion in this group is generally rapid. However, for a related temperature, it seems to be slow in the eel, which is perhaps a characteristic of the apod teleosts. The addition of iodine to the environment accelerates the excretion (platyfish, rainbow trout). The tem-

TABLE 6
I¹³¹ EXCRETION IN FRESH-WATER TELEOSTS

Species	Tempera- ture (°C.)	Treatment	Excretion of injected I ¹³¹ (%)	
			24 hours	72 hours
Goldfish				
<i>Carassius auratus</i> ⁴⁵	22-24	0	65	—
<i>C. auratus</i> ⁴⁵	22-24	Radioiodinated human serum albumin	13	—
Platyfish				
<i>Xiphophorus maculatus</i> ⁴²	?	0	40	60
<i>X. maculatus</i> ⁴²	?	Iodide-enriched water (60 ppm* KI)	78	100
Swordtail				
(<i>Xiphophorus montezumae</i>) ⁴³	?	0	50	60
Eel				
<i>Anguilla anguilla</i> L.†, ^{118, 120}	24	0	18	34
<i>A. anguilla</i> L.†, ^{118, 120}	24	Hypophysectomized	4.3	9.3
<i>A. anguilla</i> L.†, ^{118, 120}	24	Hypophysectomized and TSH-treated	8.6	16.6
<i>A. anguilla</i> L.†, ^{118, 120}	6	0	3.7	11
<i>A. anguilla</i> L.†, ^{118, 120}	6	Hypophysectomized	4.8	8.2
Salmon (<i>Salmo salar</i>)				
Parr†	14	0	26	39
Smolt†	14	0	43	—
Trout				
<i>Salmo fario</i> †	14	0	24	28
<i>S. gairdnerii</i> †	20	0	33.5	—
<i>S. gairdnerii</i> †	20	Iodide-enriched water (60 μg. I ⁻ /1000 cc.)	68.4	—

* Parts per million.

† Unpublished data.

perature seems to be an important factor, for at 24° C. the eel excretes much more than at 6° C. Hypophysectomy decreases the excretion in the eel, which accords with the report of Albert *et al.*,⁴⁴ who observed a decrease of the renal clearance in the hypophysectomized rat. The administration of TSH to the hypophysectomized eel at 24° C. increases excretion, but does not have a marked effect on the normal animal. Excretion is increased in the young salmon during the transformation from parr to smolt. Finally, an interesting fact was reported by Chavin:⁴⁵ the excretion of iodides is much slower in the goldfish when I¹³¹ is administered in the form of tagged albumin. This result is in favor of the previously mentioned hypothesis that the binding of iodine in certain migratory teleosts considerably reduces loss.

Lungfishes

The excretion is relatively slow in the lungfish maintained at 25° C., averaging 18 per cent in 24 hours. It is extremely low during estivation (unpublished data).

Amphibia

The excretion was studied after injection of I^{131} in the tadpoles of *R. pipiens*⁴⁶ at room temperature, and of *H. regilla*⁴⁷ at 22° C. In the first species 80 per cent of the dose is excreted in 7 days and, in the second, 53 per cent in 5 days. In addition, the administration of antithyroid drugs retards, as in the dogfish, the excretion of iodine in *R. pipiens*. Hunt and Dent⁴⁸ measured at 20° C. the excretion of I^{131} in tadpoles (*H. versicolor*) that had spent 24 hours in the radioactive solution. The excretion is very rapid and reaches 90 per cent in 5 days. The concentration of I^{127} in the water or the quantity of food ingested does not seem to modify the excretion pattern. All these results indicate a rapid excretion of I^{131} in the tadpole.

Reptiles

In the turtle *Terrapene carolina* Shellabarger *et al.*¹⁹ have shown a considerable variation in the excretion, depending upon whether the animal is kept in a humid or dry environment. In the first case, the excretion attains 45 per cent of the dose in 9 days; in the second case it is extremely low because the iodides accumulate in the urinary bladder where they are progressively reabsorbed into the blood.

Intrathyroidal Metabolism of Iodine

In mammals the intrathyroidal metabolism of iodine may be outlined⁵ as follows.

- (1) The thyroid gland concentrates the circulating iodides.
- (2) Iodides are oxidized and incorporated in the molecules of tyrosine, probably linked in the molecule of thyroglobulin, to give MIT and DIT.
- (3) The coupling of two molecules of DIT or of one molecule of MIT and one of DIT leads to the formation of T_4 and triiodothyronine, T_3 .
- (4) The thyroglobulin is hydrolyzed by a protease that liberates iodinated aminoacids. T_4 and T_3 pass into the blood, MIT and DIT are deiodinated by a deiodinase, and the liberated iodide is probably reutilized for hormonal synthesis.

Even though the anatomic aspect of their thyroid is very variable (simple epithelium of the endostyle of the ammocoete, dispersed follicles in the adult cyclostomes and in the majority of teleosts, and individualized glands in the other classes), the outline for mammals can be applied to the lower vertebrates in the following respects.

- (1) The thyroid of all lower vertebrates concentrates iodides, and its concentrating ability is superior to that of other tissues having the same affinity.
- (2) By chromatographic study (in part, unpublished data) of saline extracts of the endostyle of the ammocoete and the thyroid of adult lampreys, different teleosts,¹⁰ and toads,⁴⁹ it was observed that 24 hours after the injection of I^{131}

the greater part of the radioactivity is localized at the origin, suggesting a thyroglobulinlike substance. In hydrolyzates of the thyroid of cyclostomes, selachians, teleosts, lungfish, Amphibia, and reptiles a short time after the injection of I^{131} the presence of MIT and DIT was shown. After a longer period of time T_4 and, frequently, T_3 were demonstrated.* The species studied were recently reviewed by Berg *et al.*⁵⁰

(3) The existence of a protease has been demonstrated in the endostyle of the ammocoete and in the thyroid of the adult lamprey and of the dogfish.^{51, 52} Furthermore, the existence of free T_4 has been shown in the nonhydrolyzed extract of the endostyle of the ammocoete and the thyroid of the adult lamprey¹⁰ (in part, unpublished data).

(4) The existence of a deiodinase has not been reported, but the fact that only T_4 and T_3 have been found in notable quantities in the plasma of the ammocoete, lamprey, trout, and lungfish^{10, 37} (in part, unpublished data) is in favor of it.

Even though the biochemical processes of thyroid function seem well established there is still the controversial problem of the area of production of the organic binding of iodine in the thyroid follicles, that is, colloid or epithelial cells. The first hypothesis is based on the autoradiographic studies of Nadler and Leblond⁵³ and of Wollman and Wodinsky,⁵⁴ and the localization of the iodides in the colloid after antithyroid treatment.^{55, 56} However, certain authors have found organically bound I^{131} in epithelial cells.^{57, 58} Certain autoradiographic facts obtained in the lower vertebrates seem in favor of this last. It is known that in the ammocoete there are no thyroidal follicles, but that iodine is trapped by certain epithelial cells of the endostyle.⁵⁹ Olivereau⁶⁰ and Barington and Franchi,⁶¹ by fine autoradiography, have shown clearly the presence of organically bound iodine in the supranuclear region of the type III epithelial cells and at their apical surface. These epithelial cells present a great advantage for autohistoradiographic study because of their great height,⁶⁰ 30 to 35 μ . Loci of organically bound I^{131} have been observed equally in the dogfish embryo,⁶² in the tadpole of *H. versicolor*,¹⁸ and in the tadpole of *Xenopus*,⁶³ although the thyroid is uniquely constituted of cellular cords without differentiated follicles. Saxén *et al.*⁶³ relate the appearance of the organic binding in *Xenopus* to the presence of the first intracellular colloid droplets. The addition of these facts to those obtained in the chick by Trunnell and Wade,⁶⁴ who noticed that the formation of MIT and DIT occurs before the appearance of the colloid and that T_4 appears simultaneously with the first droplets of intracellular colloid, indicates that the organic binding of iodine and even the synthesis of T_4 may occur in the epithelium. A primitive process is not excluded, however, and when the follicles are constituted and accumulate colloid the organic binding might take place at the periphery of the colloid, near the colloid-cell junction.⁵³

The hypothesis of an unusual feature of the endostyle of the ammocoete has been presented by Gorbman.^{4, 65} According to him, the iodinated thyroglob-

* Unknown radioactive compounds in the thyroid of teleosts and reptiles have been reported by different authors,⁵⁰ but it is not permissible to attribute a hormonal role to these compounds, as they have not been characterized and one can consider that the thyroid hormones are identical in all the series of vertebrates.

ulin secreted by the epithelial cells is released in the lumen of the endostyle and, by the endostylar duct, in the pharynx and intestine where it is hydrolyzed by intestinal enzymes. Absorption of the thyroid hormone takes place through the digestive epithelium. If it is true that the secretion of the endostylar cells is released in the lumen,^{59-61, 66, 67} (historadiography shows radioactive masses in it), it still cannot be concluded that the hydrolysis of thyroglobulin is not realized in the endostyle. Furthermore, the presence of a protease and of free T_4 in this structure permits the supposition that hydrolysis takes place in the endostyle and that the liberated thyroid hormones are carried away in the pharynx with the mucous secretions of the endostyle, to be absorbed by the digestive wall. This hypothesis seems supported by the work of Barrington and Franchi,⁶¹ who suggest that the iodinated product leaves the cell in an uncolorable form, while the thyroglobulin is colored. The transformation is possibly produced in a zone near the cell apex; this zone stains blue with Azan.

If the biochemical function of the thyroid of lower vertebrates (outside of possible variations in the anatomic localizations of the processes indicated above) seems qualitatively identical to that of all vertebrates, quantitatively there exists a considerable variation in the speed and maximal value of the uptake of I^{131} in lower vertebrates.⁶⁰ Thus it is sometimes difficult to determine thyroid activity from maximum figures. Indeed, the fixation of I^{131} depends on numerous factors, that is, content of I^{127} in the interior and exterior environments, rapidity of radioiodide excretion, and affinity of tissues other than the thyroid for iodide. We have demonstrated above that these factors vary greatly from one species to another, or from one zoological group to another. Thus only a low uptake of iodine is not necessarily an index of thyroid hypoactivity, and the accumulation of I^{131} is not always a good index of thyroid activity, as already stated by Berg *et al.*⁵⁰ The problem is complicated further by the presence, in certain species of teleosts, of functional thyroid tissue in the head, kidney, and other tissues,^{45, 68, 69} tissues that can accumulate I^{131} in quantities equal to or greater than the quantity fixed by the normal thyroid.⁴⁵ In spite of these important difficulties, one can state with Berg *et al.*⁵⁰ that, in general, when the speed of accumulation is rapid, the turnover, or loss of newly metabolized iodine, is similarly rapid. However, this rapidity of turnover, although an index of activity of the gland, does not indicate the quantity of stable hormone actually secreted.

We present below, for each class or order studied, examples of iodine metabolism.

CYCLOSTOMES

In the lamprey the uptake of I^{131} in the gland is always very low. For a water temperature of 13 to 15° C., it does not exceed 0.36 per cent of the dose in the female *P. marinus* or 0.53 in the male.¹¹ In the ammocoete of *L. planeri*, at 20° C. the maximal uptake is 1.15 to 2.6 per cent, and in the maturing adult, 1.15 per cent (FIGURE 3)¹⁰ (in part, unpublished data). In the ammocoete of *P. marinus* at 15° C. fixation after 48 hours is 0.47 per cent.⁷⁰ This low uptake is due to 2 principal factors, (1) the affinity of the notochord and, in the adult,

of the ovary, for iodine, and (2) the small volume of glandular tissue (simple epithelial cells in the ammocoete; approximate weight of 3 mg. for the thyroid of the marine lamprey weighing 800 gm.*). However, the turnover of labeled iodine is very rapid, since the maximal fixation is attained in 6 hours in the ammocoete or adult of *L. planeri* and in 24 hours in *P. marinus*. A rapid decrease of the fixed iodine is then observed, corresponding to the liberation of the hormone in the blood, which appears at about 6 hours in the ammocoete and adult of *L. planeri* at 20° C., and at 72 hours in the marine lamprey at 13 to 15° C.

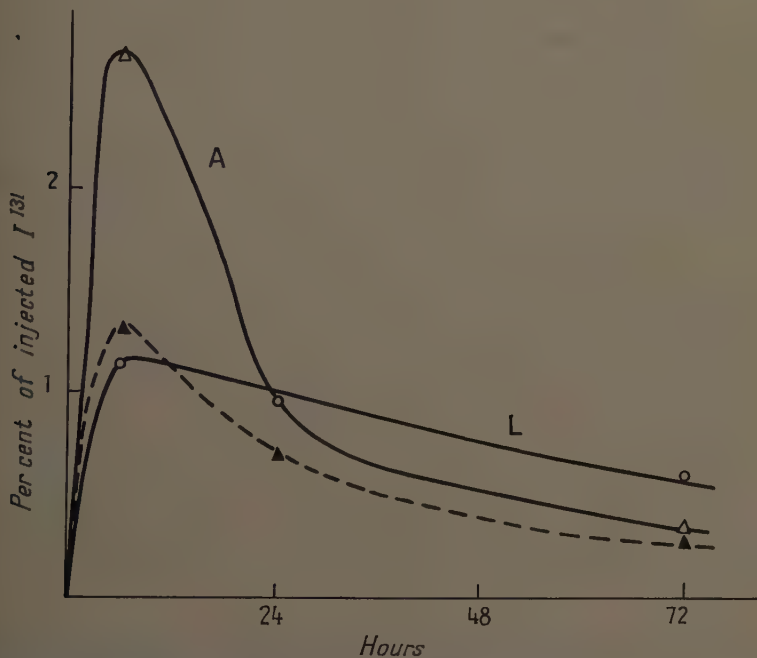


FIGURE 3. Uptake of I^{131} by endostyle of ammocoete (A) and thyroid of lamprey (L) (*Lampetra planeri*). Dotted line, I^{131} organically bound in endostyle.

The comparison of iodine metabolism in the ammocoete and in the adult of *L. planeri* is particularly interesting because, unlike *L. fluviatilis* or *P. marinus*, the adult is nearly the same size as the larva. Although the maximal fixation in the endostyle is higher than that observed in the thyroid of the adult, the organic binding is of the same type (FIGURE 3). Indeed, as has been pointed out before,¹⁰ the iodide represents an important part of the endostylar radioiodine (50 to 63 per cent after 6 hours, 25 per cent after 72 hours), while in the adult the percentage of iodide is small (7 to 10 per cent whatever the time). This higher content of labeled iodine of the endostyle seems to result from a slow disappearance of I^{131} from the plasma of the ammocoete, probably following the absence of the ovary. This significant plasma radio-

* Evaluated by measurement of follicular islets in serial section (Olivereau).

activity produces a greater amount of radioactivity in the extracellular spaces of the endostyle than in those tissues containing adult thyroid. However, as the percentage of iodine is still elevated in the endostyle 72 hours after injection, while the plasma radioactivity is relatively the same as in the adult, it is tempting to postulate that some cells of the endostyle already are able to fix iodides but not to bind them organically. These cells may be of type IV, which constitute in the large majority, at metamorphosis, the thyroid follicles of the adult.

Paper chromatography, aside from the significant presence of iodide in the ammocoete, does not show any important difference in the distribution of iodine between larva and adult. The percentage of T_4 is smaller in relation to the total radioactivity of the ammocoete, but of the same order in relation to the organic iodine.

An exhaustive study of iodine metabolism in the ammocoete during metamorphosis has not been made. However, the study of one ammocoete during metamorphosis shows, in relation to the true ammocoete, a significant reduction in the uptake of I^{131} of 8 times less (0.25 per cent in metamorphosis and 2 per cent in the true ammocoete, 6 hours after injection). The blood radioactivity is identical in both cases (unpublished data). The proportion of inorganic iodine, measured by trichloracetic precipitation, significant in both cases, seems to indicate (although a histological study has not been made) that the thyroid follicles were not differentiated. As to the proportion of T_4 , it is low and seems little different in the 2 phases of the animal, but in absolute values it is much lower in the animal during metamorphosis. Metamorphosis therefore seems to be accompanied by a considerable reduction in iodine-concentrating ability, although a complete study is necessary to confirm the first observations. The decrease in thyroid activity during metamorphosis may be related to the unsuccessful attempts at induction of metamorphosis in the ammocoete by administration of iodine, T_4 , or thyroid extract.⁷¹⁻⁷⁵

SELACHIANS

The intrathyroidal metabolism of iodine was studied in the dogfish *S. stellaris*,⁷⁶ and particularly in *S. canicula*.^{39,76,77} According to the observation of Leloup,⁷⁶ the fixation is extremely low in the thyroid, not more than 0.45 per cent of the dose 3 days after the injection. However, Gorbman *et al.*⁷⁷ observed a very rapid and elevated uptake, the maximum after 6 hours being 20 to 34 per cent of the dose. The I^{131} of the thyroid rapidly decreases and represents only 0.13 to 0.21 per cent of the dose after 144 hours. The authors emphasize the important variation observed in the uptake for the same time after the injection. By chromatography, with the thyroid hydrolyzate in butanol acetic acid solvent (BAC), they have shown the disappearance of all inorganic iodine after 24 hours and the appearance of T_4 after 17 hours, which represents 53 per cent of the I^{131} remaining in the gland after 96 hours. DIT disappeared after 96 hours, while MIT remained in small quantities.

Because of the difference between our observations and those of Gorbman *et al.*,⁷⁷ we recently restudied iodine metabolism in the dogfish at 20° C. by chromatography with BAC and pentanol/ NH^3 solvents. We have observed

again that the uptake of I^{131} in the thyroid is very low and that the curve obtained is similar to the one reported in 1952 (FIGURE 4). The maximal uptake is observed after 24 hours and the decrease of iodine is very slow. The chromatographic study shows that T_4 may appear after 24 hours (in the animal with greatest uptake) and is always present after 72 hours, but in small quantities (maximum, 6 per cent). In one case, we have found T_3 after 72 hours. All these facts seem to indicate that the turnover of iodine in the dogfish is very slow. Contrary to Gorbman *et al.*,⁷⁷ we observed the presence of significant quantities of iodine, even 72 hours after injection. Finally, the quantities of MIT and DIT vary little with time. In the intervals studied, no hormonal iodine appeared in the plasma. The only difference between the experimental procedures of Gorbman *et al.*⁷⁷ and of ours resides in the geographical origin of the animals, Atlantic dogfish in the first case, Mediterranean in the second, but it does not seem to account for the discrepancies observed.

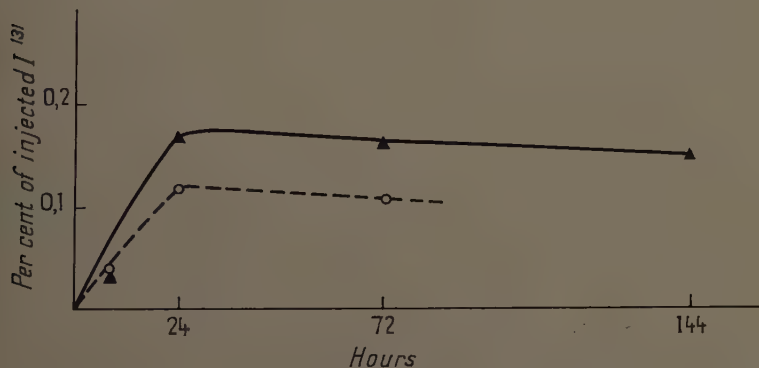


FIGURE 4. Uptake of I^{131} by thyroid of dogfish (*Scyllium canicula*). Line, experiment 1952; dotted line, experiment, 1959.

TELEOSTS

The order of teleosts is probably the most studied from the point of view of thyroid function in lower vertebrates, especially by the school of Gorbman in New York and Fontaine in Paris. The maximal uptake is generally less in the marine teleosts than the fresh-water teleosts. Actually, maximal uptakes less than or equal to 4 per cent have been cited in the mullet, the conger eel⁷⁸ at 16 to 19° C., the starry flounder (*Platichthys stellatus*),⁷⁹ and an uptake of 10 per cent in *Fundulus majalis*⁸⁰ at 22° C. In the fresh-water teleosts fixation is clearly more important at similar temperatures^{50,81} or even at lower temperatures.⁸²⁻⁸⁵ However, some fresh-water species seem to have a very slow intrathyroidal metabolism, such as the sunfish,⁵⁰ the goldfish (*Carassius auratus*), which accumulates 3 per cent of the iodine in the thyroid⁸⁶ and, in particular, another cyprinid, the adult *Cyprinus carpio*, whose uptake is less than 1 per cent (unpublished data). Moreover, it is necessary to specify that most of the I^{131} present in the thyroid of the carp is in the inorganic form and that a significant fraction is probably in the nonthyroidal tissue associated with the glandular tissue. This extremely slow iodine me-

tabolism coincides well with the histological aspect of the thyroid, which is composed of extremely dispersed follicles nearly at complete rest.⁸⁷

The chromatographic study of thyroid hydrolyzates permits the following remarks. Inorganic iodine often exists in significant quantities, principally immediately after the injection, but it may persist for a long time, especially at a low temperature. MIT and DIT are in general the most important constituents. The proportions of T_3 and T_4 appear variable. Berg *et al.*⁵⁰ have found as much as 70 per cent of T_4 in *F. heteroclitus*. The thyroid of *Umbra* can synthesize either T_3 or T_4 , or the two together. The calculation, according to the authors' data,⁵⁰ shows that when T_4 alone is present, the MIT:DIT ratio equals 1, while it is 3 when T_3 alone is produced. These variations are further evidence of the correlation in the rat and the *Periophthalmus* between the MIT:DIT ratio and the T_3 : T_4 ratio.^{88,89} In general, T_3 is found in the most active glands of the *Periophthalmus*,⁸¹ *Fundulus*, *Notropis deliciosus*,⁵⁰ and rainbow trout (unpublished data) treated with TSH.

In certain species the proportion of T_3 and T_4 can be small and difficult to show. This fact can be due to the slow iodine metabolism (as in *C. auratus* and *Lepomis gibbosus*⁵⁰) and also to the rapid excretion of hormones into the blood (as in the *Periophthalmus*).⁸¹ Small quantities of T_3 and T_4 have been found in the very active thyroid of different species of vertebrates (lamprey, *Protopterus*,³⁷ little dormouse⁹⁰) while the plasma contains significant quantities of these hormones. The presence of small quantities of T_3 and T_4 in the thyroid therefore is not necessarily an indication of a slow hormonal biosynthesis. Some examples of iodine metabolism in teleosts will be given in the section on the influence of temperature and migratory behavior.

LUNGFISHES

The only species studied, *P. annectens*, demonstrates thyroid function that is very intense,³⁷ and that will be discussed later.

AMPHIBIA

Anura

All authors observe a relatively low uptake of I^{131} in the adult *Anura*. In the male summer frog (*R. pipiens*), the maximal uptake, reached after 48 hours, is from 4 to 5 per cent of the injected dose.⁹³ It is 7.5 per cent after 96 hours in *R. temporaria* several weeks before egg-laying.¹³ At 12° C. the thyroid of *R. esculenta* contains 1.3 to 5.5 per cent of the dose 8 days after injection (unpublished data). Finally, in the toad *B. arenarum* at 22° C. the low uptake after 24 hours increases until the fourth day after the injection and attains 9.6 per cent of the dose.⁴⁹

The variations of the metabolism of I^{131} in the tadpole in the course of metamorphosis are reviewed later.

MIT, DIT, T_3 , and T_4 have been characterized in the thyroid of *R. pipiens* and *X. laevis*, tadpole and adult.^{50,92} In *B. arenarum* the iodides always represent less than 5 per cent of the total radioactivity of the gland. The percentage of DIT is greater than that of MIT, while that of T_4 attains a maximum of 17 per cent, 96 hours after injection.⁴⁹

Urodela

In two neoteinic adult Urodela, *Amphiuma means* and *Necturus maculosus*, the maximal uptake is small at 20° C., less than 1 per cent in the first, and 1.5 per cent in the second.⁵⁰ There is neither T₃ nor T₄, 96 and 192 hours after the injection. Unfortunately, it is difficult to interpret these results because the experimental animals were treated shortly before the injection of I¹³¹ with pellets of T₄ that may have helped retard the entry of radioiodine in the gland and the biosynthesis of hormones. However, in another neoteinic salamander, *Eurycea*, the accumulation of I¹³¹ does not exceed 2.5 per cent of the dose, and the half life is 7 days (Gorbman and Dundee, cited by Kaye and LeBourhis⁴⁷). The iodine metabolism in neoteinic Urodela thus seems to be slow.

REPTILES

The only intensive study is that of Shellabarger *et al.*¹⁹ on turtles, that is, a land species, *T. carolina*, and an aquatic species, *P. floridanus*. The I¹³¹ uptake is elevated at 21 to 23° C. when the animals are maintained in a dry place, the maximum being 80 per cent. In a wet environment the maximal uptake is not more than 15 per cent in *Terrapene*. The accumulation of I¹³¹ in the urinary bladder (where it is progressively reabsorbed) in the animals in a dry environment explains this difference. MIT, DIT, T₄, and traces of T₃ were shown to be present.

In 2 species of lizards, *Anolis carolinensis* and *Sceloporus occidentalis*, in a dry environment the maximal uptakes are 12 and 25 per cent, respectively. T₄ represents about 10 per cent of the thyroidal I¹³¹, and traces of T₃ exist (Kobayashi and Gorbman, cited by Berg *et al.*⁵⁰).

Circulating Thyroid Hormone

NATURE

While the thyroid of lower vertebrates has been the object of numerous studies, the study of the circulating hormone has received less attention. T₄ has been characterized in the plasma of the ammocoete¹⁰ and the adult of *L. planeri*, the rainbow trout, *S. gairdnerii* (unpublished data), *Periophthalmus*,⁸¹ and the lungfish.³⁷ T₃ found in the same species exists in lesser quantities than T₄.

PLASMA CONTENT OF HORMONAL IODINE (I¹²⁷)

It has been determined only in cyclostomes, selachians, teleosts, and lungfish (TABLE 5). Similar to iodides, the plasma content of hormonal iodine is very variable. In the marine lamprey and the lungfish it is of the same quantity as in mammals and birds. In fresh-water and marine teleosts elevated values are observed in some species and low values in others. High values are usually found in migratory species (mackerel, mullet), but it is the typical amphibiotic migrators (salmon, sea trout, shad) that show the greatest content in hormonal iodine, as they have also the greatest content⁸¹ of iodide. It is probable that this peculiarity is related to the intense muscular activity manifested by these fish in upstream swimming. We have shown recently

that the upstream battle increases the need for thyroid hormone of the rainbow trout.⁹³

BINDING OF THYROID HORMONES TO PLASMA PROTEINS

The existence of a thyroxine-binding protein (TBP) has been well demonstrated in several species of mammals. Apter *et al.* (quoted by Robbins and Rall⁹⁴) studied the serum of two reptiles, the crocodile and the alligator. These two species have two proteins binding T_4 , of which one is albumin, but it is not certain that they possess a specific TBP. Recently Tata (personal communication) demonstrated the existence of TBP in two species of teleosts: plaice and brown trout.

DISTRIBUTION OF THYROID HORMONES T_3 AND T_4 BETWEEN RED BLOOD CELLS AND PLASMA

This distribution has been studied in the carp, eel, salmon, and lungfish,²⁵ and it varies with the species (TABLE 7). T_4 does not penetrate into the RBC

TABLE 7
H/P RATIO OF I^{131} -LABELED IODIDE, TRIIODOTHYRONINE, AND THYROXINE IN SOME TELEOSTS

Species	H/P ratio		
	Iodide	Triiodothyronine	Thyroxine
Carp	0.43	0.41	0.14
Eel	0.53	0.19	0.07
Salmon (adult)	0.06	0.17	0.15

of the eel or the lungfish, but penetrates into those of the salmon ($H/P = 0.15$) and carp ($H/P = 0.14$). The case of the salmon is particularly remarkable since the H/P is higher for T_4 than for iodide. With T_3 the H/P is always higher than with T_4 , but the difference is small in the case of the salmon. However, in the carp T_3 penetrates into the RBC as do iodides. If one agrees with Crispell *et al.*⁹⁵ that the factor limiting the penetration of T_4 in the RBC of mammals is the specific link with a plasma protein, the facts summarized in the foregoing seem to indicate that in certain species of fish, T_4 and, particularly, T_3 are not completely bound to plasma proteins.

DISTRIBUTION AND METABOLISM OF THYROID HORMONES

Teleosts

The distribution and metabolism of T_3 and T_4 labeled with I^{131} have been studied in the salmon (unpublished data) and those of T_4 in the rainbow trout and the normal and hypophysectomized eel (unpublished data).

The ratio of radioactivity per gram of tissue to the radioactivity per gram of plasma (R) for different tissues, 24 and 72 hours after the injection of labeled T_4 into the migrating adult salmon, is presented in TABLE 8. To correct

for the interference of small quantities of labeled iodide contaminating the injected hormone and iodide from the metabolism of T_4 , the radioactivity in the tissues is related to the PBI^{131} of the plasma. R is greater than 1 for the liver, bile, intestine, and thyroid at the 2 considered intervals, and for the ovary and kidney after 72 hours. The thyroid concentration, which increases greatly from 24 hours to the third day, is explained by the uptake of free labeled iodide released by the deiodination of T_4 . It is the same for the ovary, which does not concentrate T_4 ($R < 1$, after 24 hours), but accumulates iodides. Of the radioactivity of the ovary after 72 hours, 86 per cent is in the inorganic form. However, almost all of the radioactivity in the liver and intestine is bound organically. It is necessary to point out that R varies little with time in the liver, but increases considerably in the bile and intestine. Similar

TABLE 8
CONCENTRATION* OF RADIOACTIVITY IN VARIOUS TISSUES OF SALMON
AFTER INJECTION OF LABELED THYROXINE

Tissue	24 hours		72 hours	
	Tissue/plasma ratio	I^{131} organic (%)	Tissue/plasma ratio	I^{131} organic (%)
Ovary	0.24	67.5	4.71	14.2
Liver	1.66	98	1.95	95
Bile	150.4	—	606.6	—
Intestine	1.28	91	5.86	85
Kidney	0.54	85	1.88	81
Muscle	—	—	0.31	71
Thyroid	6.86	94.5	164.4	98
Pituitary	0.55	—	0.82	—
Hypothalamus	0.12	93	0.71	80
Plasma	1.00	82	1	41

* Expressed as the ratio of the radioactivity per gram of organ weight to the trichloroacetic insoluble radioactivity per gram of plasma.

results have been obtained in the trout and eel and seem to show the existence in teleosts of an enterohepatic cycle comparable to the one observed in mammals.

In the young salmon, T_3 is metabolized more rapidly than T_4 and its bile concentration is more elevated. A more rapid disappearance of T_3 has been found equally in man and different mammals.⁵ The temperature distinctly influences the metabolism of T_4 in the eel. At 24° C., T_4 disappears more quickly from the blood and is deiodinated more rapidly than at 6° C. Hypophysectomy does not seem to affect the speed of utilization of T_4 , contrary to the results obtained with the rat.⁹⁶

Amphibia

Schmidt,⁹⁷ after injecting I^{131} in the salamander *Ambystoma gracile*, found in the bile 4 days later, in addition to iodides, iodinated compounds of which one is probably T_4 .

Volpert *et al.*¹³ have shown that the ovary of *R. temporaria* does not concen-

trate T_3 or T_4 , but their concentration in the skin is 5 or 6 times greater than that in the plasma. The question of a selective concentration of thyroid hormone by the frog's melanocytes merits investigation. A small concentration of T_4 in the pars intermedia of the pituitary of *R. esculenta* has been pointed out recently.⁹ This fact is perhaps consistent with the inhibition of uptake of I^{131} in the thyroid of the frog after treatment with intermedin.¹⁵

DEGRADATION OF THYROID HORMONES

The presence, in the plasma of teleosts that have received labeled T_3 or T_4 , of a proportion of labeled iodide increasing with the time interval seems to indicate that one of the important processes of degradation of thyroid hormones in fishes is deiodination. This hypothesis is supported by the recent demonstration of the presence of a deiodinase in marine and fresh-water fishes (Tata, quoted by Pitt-Rivers and Tata⁵).

Pituitary Control of Thyroid Function

HYPOPHYSECTOMY

Hypophysectomy produces in the dogfish embryo,⁶² the eel,⁹⁹ the goldfish,⁴⁵ and the toad⁴⁹ a marked decrease in the thyroid uptake of I^{131} in comparison to that in normal animals, 5 to 8 days after operation. In the eel the reduction of I^{131} uptake is accentuated with time, although the histological aspect of the gland is not characteristically modified. The organic binding of iodine is considerably retarded because the percentage of inorganic I^{131} in the gland is always considerable. The synthesis of T_4 and its liberation into the plasma are equally slow. Hypophysectomy in the eel therefore seems to depress the different phases of intrathyroidal I^{131} metabolism in the same manner as in the rat.¹⁰⁰ The only difference seems to be a slower evolution of these processes in the eel, probably due to the influence of temperature.

INFLUENCE OF TSH

Cyclostomes

Histological studies seem to indicate pituitary control of the thyroid activity of the endostyle of the ammocoete. In fact, TSH injections induce hypertrophy of type III and type V cells (Gorbman, quoted by Goldsmith¹⁰¹ and Oliveureau¹⁰²). An analogous result is observed after treatment with antithyroid drugs.^{102, 103}

Autoradiographic study¹⁰² or biochemical study (unpublished data) of I^{131} metabolism in the ammocoete of *L. planeri* treated with TSH at 20° C. does not show an increase of I^{131} uptake in the endostyle 6 or 24 hours after its injection. Nevertheless, TSH treatment accelerates the disappearance of plasma I^{131} . The plasma radioactivity in treated animals is 2 times less after 6 hours and 1.5 times less after 24 hours than that in controls. As a result, the ratio I^{131} per gram endostyle/ I^{131} per gram plasma is 2 times higher after 24 hours in the treated animals. This difference disappears after 48 hours. TSH therefore influences the thyroid function of the endostyle, but its action

is relatively weak and transitory, owing to the intense activity of the endo-style of the control animals.

Selachians

One, 3, or 6 daily injections of TSH in *S. canicula* increases the fixation of I^{131} in the thyroid at 15 to 16° C., and the thyroid:serum ratio (I^{131} per gram of thyroid/ I^{131} per gram of blood). The action is more pronounced when the treatment is continued (unpublished data).

Teleosts

Numerous authors have shown an increase in the uptake of I^{131} in the thyroid of various marine and fresh-water teleosts after administration of TSH: conger⁷⁸ at 15 to 16° C., *C. auratus*^{45,86} at 23 to 24° C., rainbow trout, *S. gairdnerii*^{84,85,104} (in part, unpublished data) at 20 to 21° C., and the normal and hypophysectomized eel¹⁰⁵ (in part, unpublished data) at 25° C. The augmentation of the uptake of I^{131} is proportionately greater in the hypophysectomized animal (*C. auratus*,⁴⁵ and unpublished data on the eel) (FIGURE 5), or where the pituitary-thyroid axis is put at rest by the addition of iodinated casein to the diet or by fasting (rainbow trout^{84,85}).

In *C. auratus*⁸⁶ or the rainbow trout (unpublished data), the proportion of iodides, MIT, and DIT in the gland is not particularly modified, but T_4 is distinctly increased. In the normal eel, at 25° C., TSH accelerates the organic binding of iodine and the synthesis of T_4 soon after the injection of I^{131} . The same phenomenon, but much more accentuated, is observed in the hypophysectomized animal. In this case TSH not only restores normal function but accelerates the process of iodine organification and the synthesis of T_4 .

TSH produces discharge of the hormone stored in the thyroid of the normal and hypophysectomized eel at 25° C., as indicated by the decrease of thyroidal I^{127} (FIGURE 6). The effect, distinct 24 hours after the termination of the treatment with TSH and the injection of I^{131} , reaches its maximum in 3 days. The thyroid iodine returns to the normal amount in 6 days in the controls, but not in the hypophysectomized eel, where the thyroid iodine is more elevated than in the normal eel (unpublished data).

Amphibia

Donoso and Trivelloni,⁴⁹ after injection of the pars distalis of the toad at 22° C., observed an increase of 56 per cent of the I^{131} uptake in the thyroid of the same species over that of normal animals. D'Angelo¹⁰⁶ also observed in the tadpole an increase in the I^{131} uptake after TSH treatment. He observed, however, after injection of a large dose of TSH, a decrease in thyroid radioactivity that later returned to the normal level.

Reptiles

TSH increases the rate of thyroidal accumulation and, possibly, the release of I^{131} in the turtle *T. carolina* maintained at 21 to 23° C. The female was somewhat less responsive than the male.¹⁹

*Influence of Temperature on Response of the Thyroid
of Lower Vertebrates to TSH*

All the experimental results reported above have been obtained in animals at temperatures of 15° C. or greater and, in general, with purified prepara-

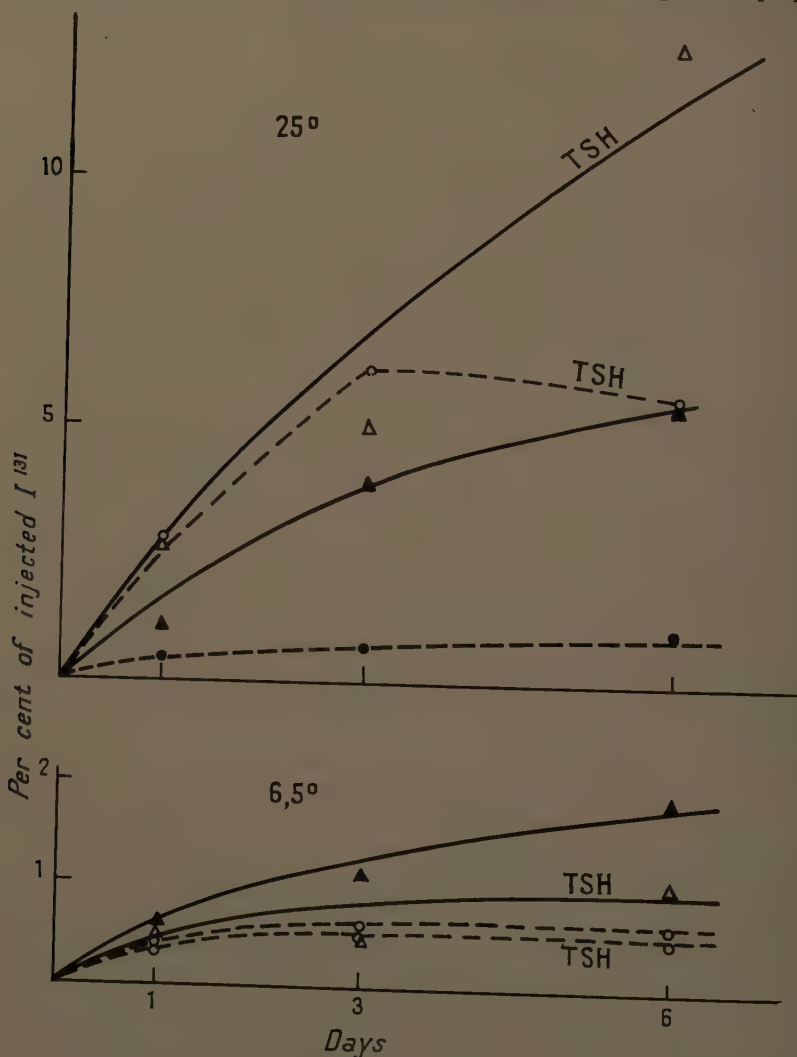


FIGURE 5. Influence of thyrotropic hormone on the uptake of I^{131} by the thyroid of normal (lines) and hypophysectomized eel (dotted lines) at 6.5° C. and 25° C.

tions of TSH of mammalian origin. At a low temperature these preparations, when administered according to the same procedure followed with high temperatures, have little effect or may inhibit the uptake of I^{131} by the thyroid in rainbow trout,^{84,104} eel¹⁰⁴ (in part, unpublished data) (FIGURE 5), and turtle.¹⁹ Such results are explained by an action of the temperature on the kinetics of

the reactions involved. Such an action in the rainbow trout was shown by Fontaine and his associates,^{84,107,108} and we have observed in this species at 14° C. that 3 injections of TSH, 24 hours apart (I^{131} being administered with the last TSH injection), produces no increase in the fixation of I^{131} after 24 hours. The action manifests itself only after 3 days and is still distinct after 6 days (FIGURE 7). An inhibition of, or no action in, the uptake of I^{131} in the mammalian thyroid has also been shown *in vivo* and *in vitro* in the first interval (12 to 24 hours) after the injection of TSH.¹⁰⁹⁻¹¹⁵ It appears logical to suppose

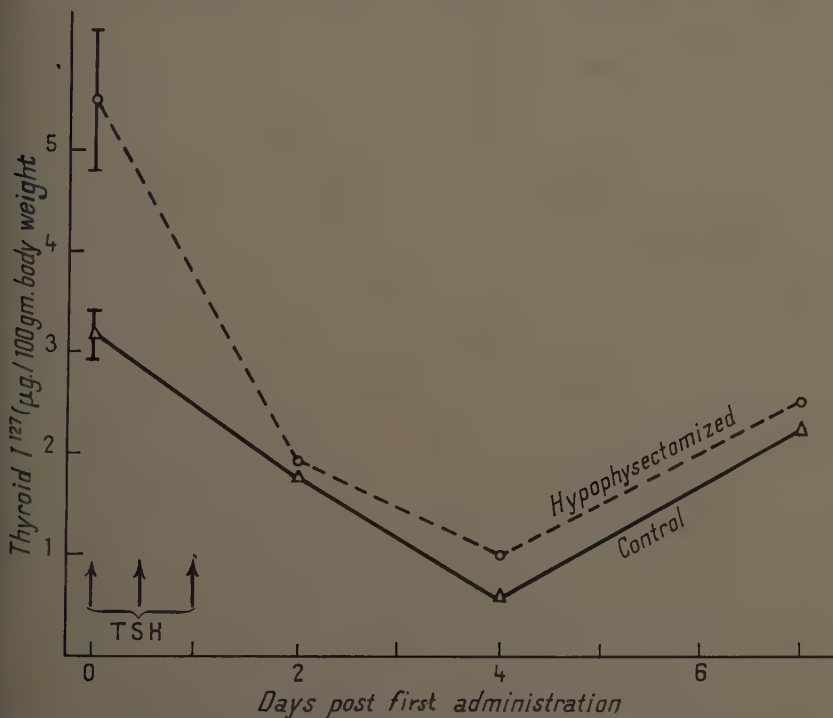


FIGURE 6. Influence of thyrotropic hormone on the thyroid iodine of normal (line) and hypophysectomized (dotted line) eel at 25° C. Vertical lines, standard error.

that this stage of inhibition will be larger as the temperature in the experiment is lower, and that after several injections of TSH the stimulation of the "iodide pump" will be evident only after a certain delay. As for the mechanism of the inhibition of the entry of I^{131} in the gland, Deiss *et al.*¹¹⁴ hypothesize that "some product of the TSH increased proteolysis of the thyroglobulin temporarily acts as an inhibitor of further utilization of iodide, i.e. an intrathyroidal feedback." This inhibition could affect the iodide "being recycled from the deiodinase reaction within the gland," and a part of this iodide may be released in the plasma. The results obtained in the normal eel treated with TSH at 6° C. seem to be in favor of this interpretation (FIGURE 5). Indeed, even 6 days after the injection of I^{131} the inhibition of its uptake, as well as its organic binding, is apparent. The content of I^{127} of plasma is greater than

that of the controls. This increase of the content of inorganic I^{127} of the plasma, by decreasing the specific activity of I^{131} in the plasma, can contribute to the apparent inhibition of the mechanism of iodine fixation.

Nevertheless, it does not necessarily follow, as might be imagined from the preceding experiments, that the stimulation of the thyroid by the pituitary is so slow in cold-water fish that it is little adapted to the metabolism and rapid reactions of certain species (*Salmonidae*, for example) and that its physiological role should be doubtful.

The results relative to the action of the temperature on thyrotropic activity are not identical when obtained from pituitary extract of mammals as compared with that of teleosts. Thus a crude extract of eel pituitary is as active on the trout at 10°C . as at 20°C ., while a crude extract of rat pituitary or a

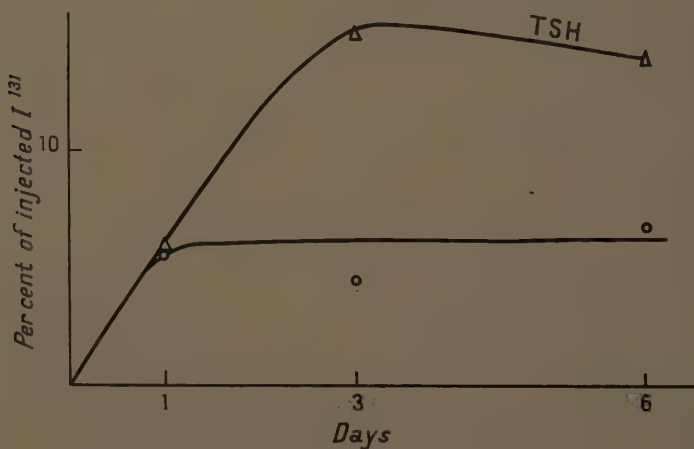


FIGURE 7. Influence of thyrotropic hormone on the uptake of I^{131} by the thyroid of rainbow trout at 14°C .

purified preparation of TSH of mammalian origin is less effective at 10°C . than at 20°C ., in spite of a slower procedure at 10°C .¹⁰⁷ This fact and others that do not pertain to the substance of this article lead to a certain zoological specificity, either in regard to structural differences or differences of physico-chemical state, between the active principle of the pituitary gland of teleosts and that of mammals.¹¹⁶

It is very likely that this zoological specificity is pertinent to other zoological groups since, by their positive action on the thyroid of mammals and their variations in activity according to the temperature, the pituitary extracts of lungfish are distinguished from those of the teleosts.¹¹⁷

Variations of Iodine Metabolism and Thyroid Function in Light of Different Ecologic and Ethologic Factors

Among the numerous ecologic and ethologic factors able to influence iodine metabolism in lower vertebrates are genital maturity, nutrition, environmental

concentration of iodine, salinity, season, photoperiod, amphibiosis, and desiccation. We shall consider only the influence of temperature and the variations of thyroid function as they are related to the migration of the salmon, the estivation of the *Protopterus*, and the metamorphosis of Amphibia.

TEMPERATURE

The influence of temperature on the thyroid function of poikilotherms studied with I^{131} is still controversial. A positive correlation between I^{131} uptake and temperature has been observed in the *Fundulus*,⁸⁰ eel,¹¹⁸ tadpole of *Xenopus*,⁶³ and turtle.¹⁹ However, by autoradiography, Olivereau¹¹⁹ observed a decrease of I^{131} organically bound in the trout thyroid at 20° C. in relation to that maintained at 10° C. Also, Gorbman⁴ states that *U. limi* seems to be like the trout and "accumulates a higher maximum of I^{131} at 12° C. than at 20° C." These last results, however, are not entirely conclusive. As we have noted in the foregoing, the value of iodine uptake in the thyroid is the result of several factors, for example, concentrating ability of the gland, radioiodide excretion, and I^{127} content of the blood. We have shown that at high temperature the excretion is increased and, in consequence, the speed of disappearance of I^{131} from the plasma is accelerated. As a result, the gland can take up less iodine even though it is more active. It is thus that in the ammocoete we have observed a higher uptake in the endostyle at 6° C. than at 20° C., while the distribution of I^{131} in the endostyle and the speed of appearance of T_4 in the blood indicate a metabolism accelerated at 20° C. However, at the different times considered, the radioactivity of the plasma is 2 to 4 times more elevated at 6° C. than at 20° C. (unpublished data). Gorbman⁴ indicates that in *U. limi* the maximum of fixation, greater at 12° C. than at 20° C., is attained more slowly at low temperatures, which seems to show a more rapid turnover at 20° C. In addition, Fontaine and Fontaine¹⁰⁷ have observed in the rainbow trout a higher uptake at 20° C., 24 hours after the injection of I^{131} , than that measured after 48 hours at 10° C. The study of the influence of temperature on the thyroid function of poikilotherms therefore necessitates a knowledge not only of the uptake of I^{131} in the thyroid but also of its intrathyroidal distribution, of its speed of disappearance from the blood, and of the content of I^{127} in the plasma and the thyroid. Such a study has been done on the normal and hypophysectomized eel.^{118,120}

In the normal eel the I^{131} uptake of the thyroid is more elevated and the maximal fixation attained earlier when the temperature is higher (FIGURE 8). Correspondingly, the organic binding of iodine and the synthesis of T_4 are accelerated by the increase of temperature. The labeled hormonal iodine appears in small quantities (9 per cent of the I^{131} of the plasma 6 days after the injection) in the eel at 25° C., while the plasma of the eel at 6.5° C. does not contain any. The thyroid-stable iodine (expressed in micrograms of I^{127} per 100 gm. of body weight) varies inversely with the temperature (TABLE 9). The content of stable iodine of the plasma decreases significantly at higher temperatures. This decrease is due particularly to the inorganic fraction of plasma iodine that is consistent with the rapid disappearance of plasma radioiodine. The PBI^{127} remains constant (TABLE 9), which indicates that the augmented peripheral uti-

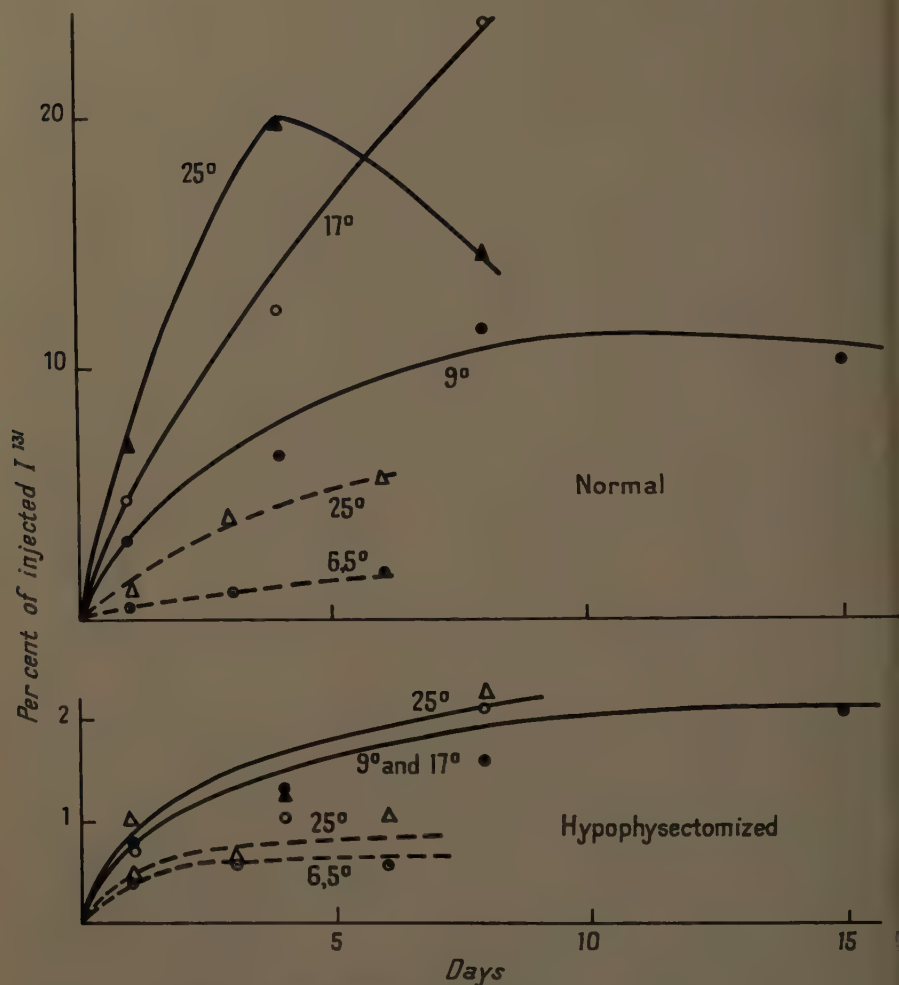


FIGURE 8. Influence of temperature on the uptake of I^{131} by the thyroid of hypophysectomized and normal eel. Lines, experiment 1; dotted lines, experiment 2.

TABLE 9
EFFECT OF TEMPERATURE ON THYROID AND PLASMA IODINE OF NORMAL
AND HYPOPHYSECTOMIZED EEL

Group	Temp. (°C.)	Thyroid iodine ($\mu\text{g.}/100 \text{ gm.}$ body weight)	Plasma iodine ($\mu\text{g. per } 100 \text{ cc.}$)	
			Total	PBI
Control	25	$3.14 \pm 0.23^*$	22.1 ± 4.9	13.1 ± 1.4
Control	6.5	5.83 ± 0.43	35.9 ± 4.3	15.9 ± 1.2
Hypophysectomized	25	5.49 ± 0.71	53.2 ± 19.3	14.2 ± 2.2
Hypophysectomized	6.5	6.61 ± 0.37	56.7 ± 8.8	22.4 ± 1.7

* Standard error.

lization of T_4 at 25° C. (previously shown) is compensated for by an increase in hormone secretion. All these facts show a large functional activity of the thyroid at high temperature.

In the hypophysectomized eel (of which the uptake at the same temperature is much less than in the normal eel) the temperature does not seem to modify the kinetics of the I^{131} uptake by the thyroid (FIGURE 8), or the respective proportions of iodides, iodotyrosines, and iodothyronines. In the plasma, I^{131} disappears very slowly, and no labeled hormonal iodine appears in the period of time considered. The thyroidal stable iodine does not vary significantly with temperature. Neither does the total I^{127} of the plasma (TABLE 9). However, the PBI is significantly lower at 25° C. than at 6.5° C. This decrease of PBI^{127} , which is not seen in the normal eel at 25° C., probably results from the increase of the peripheral utilization of T_4 at a high temperature, which is observed in the normal or hypophysectomized animal and that is not compensated for in the hypophysectomized animal by an augmentation of thyroid secretion.

Those experiments show clearly that in the eel the temperature factor acts by pituitary relay and accelerates thyroid function. Similar studies must be made before the conclusions can be applied to other species. It is possible that in certain poikilotherms a correlation as clear as the one in the eel between thyroid function and temperature does not exist.

MIGRATION

This study was done on the Atlantic salmon (*S. salar*) of the Adour Basin in France.

Young Salmon

The experiments were made in 1951 and 1955 in the springtime (temperature, 13 to 14° C.) at 3 stages of evolution: parr, sedentary; parr having more or less the silver coating of the smolt, but stopped from migrating; and smolt captured while swimming toward the sea⁸³ (in part, unpublished data).

The uptake of I^{131} is higher in the silvered parr and the smolt than in the parr, although the excretion is more rapid in the first 2 stages, as indicated by the rate constant, * k_2 (FIGURE 9 and TABLE 10). The maximal uptake is attained earlier in the smolt, indicating a rapid turnover. The ability of the thyroid to concentrate iodide, evaluated by the T/S or by the coefficient * k_1 increases from parr to silvered parr to smolt (TABLE 10).¹²¹ It is difficult to show distinct differences in the proportion of iodide of MIT and DIT, but the proportion of T_3 and T_4 is always small, not exceeding 5 per cent, and is always higher in the smolt and silvered parr than in the parr.

The thyroidal I^{127} decreases by half that of the parr in the smolt;¹²³ in the silvered parr the amount approaches that of the smolt or of the parr, depending on whether the transformation is more or less advanced. Another interesting fact is the increase of the proportion of thyroxinelike† I^{127} , which changes from

* Rate constant of thyroid accumulation of iodide = k_1 ; rate constant of renal excretion of iodide = k_2 (see Stanbury *et al.*¹²²).

† Thyroxinelike iodine is determined on the butanol extract of trypsin hydrolyzate, treated twice by 4 N NaOH. This method gives a value slightly too high.

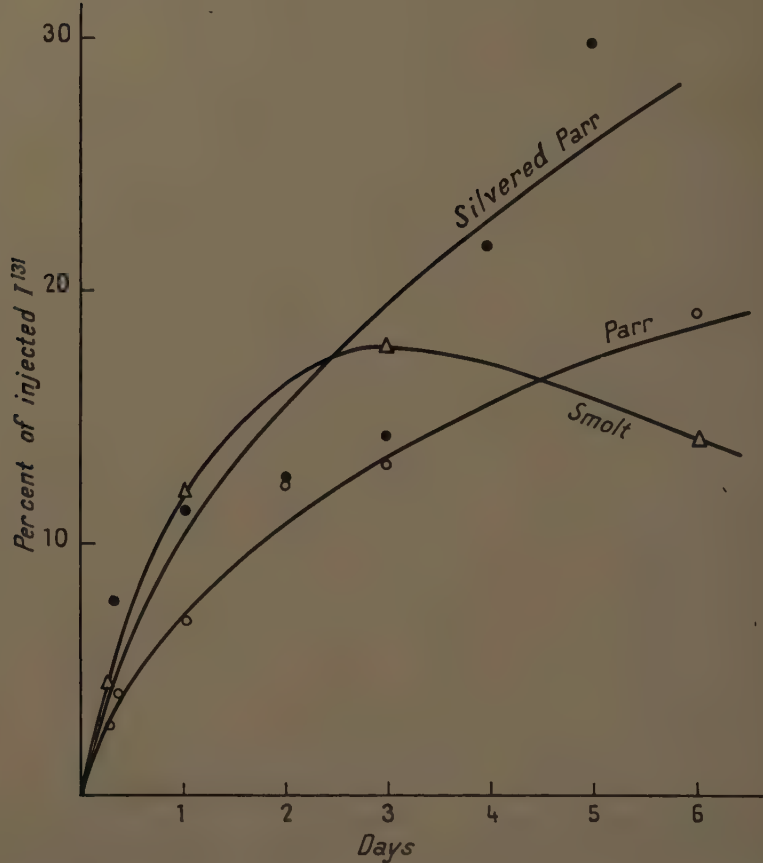


FIGURE 9. Uptake of I^{131} by thyroid of young salmon: parr, silvered parr, and migrating smolt.

TABLE 10
VARIATIONS OF T/S RATIO AND OF k_1 AND k_2 (RATE CONSTANTS OF THYROID ACCUMULATION AND OF RENAL EXCRETION OF IODIDE, RESPECTIVELY) IN YOUNG SALMON

Stage	t/S*	k ₁	k ₂
Experiment 1			
Parr	—	0.0036	0.0174
Silvered parr	—	0.0068	0.0200
Experiment 2			
Parr	4.69 ± 0.70†	0.0032	0.0108
Silvered parr	8.48 ± 1.10	—	—
Migrating smolt	9.84 ± 0.78	0.0073	0.0257

* Thyroid:serum ratio. Calculated from the equation (I^{131} thyroid/ I^{131} /100 mg. blood) × (100/ P) where P is the body weight in grams.
† Standard error.

6.9 in the parr to 8.2 in the silvered parr to 11.6 in the smolt;* the difference between the parr and the smolt is significant (TABLE 11). It seems that in the rat the proportion of thyroxinelike I^{127} increases with the activity of the gland (unpublished data). Finally, the plasma iodine is higher in the smolt than in the parr and silvered parr. This increase affects simultaneously⁸³ the inorganic iodine and the PBI. All these facts indicate an increase in the activity of the thyroid gland of the parr over that of the silvered parr, and that of the silvered parr over that of the smolt. This is in accord with the histological aspect of the thyroid at different stages,^{83,87} and with the well-known fact that the development into the smolt may be effected by the administration of thyroid hormone.¹²⁶⁻¹²⁸

The thyroïdal hyperactivity of the silvered parr and the smolt is accompanied by an increase in the thyroid secretion (*see below*).

TABLE 11
THYROID IODINE OF YOUNG SALMON

Stage	Thyroid iodine ($\mu\text{g.}/100 \text{ gm. body weight}$)		Iodothyronine percentage of total iodine, expt. 2
	Expt. 1	Expt. 2	
Parr	$10.9 \pm 0.98^*$	9.04 ± 0.66	6.9 ± 0.48
Silvered parr	$11.6 \pm 1.18^\dagger$	$5.60 \pm 1.17^\ddagger$	8.2 ± 0.29
Migrating smolt	4.1 ± 0.95	5.41 ± 0.44	11.6 ± 0.68

* Standard error.

† Not greatly silvered.

‡ Very silvered, nearly smolt.

Adult Salmon

The I^{131} uptake in the salmon is slightly higher during upstream migration (March) than at spawning (December) (FIGURE 10). So is the k_1 coefficient, which is 0.00126 in the first and 0.00088 in the second. The thyroid of the migrating salmon appears functionally more active than that of salmon on the spawning ground. Nevertheless, the difference is perhaps less important than the value of k_1 indicates, the average temperature of the experiment being slightly less in the salmon at spawning.

The plasma iodide and PBI are very high in migrating salmon and considerably lower at spawning.³⁶ Accordingly, the hormone secretion is quantitatively greater in the migrating salmon (*see below*).

ESTIVATION

A comparative study of the thyroid function of the active lungfish in aquatic environment and the lungfish in estivation (land environment), during which

* This low proportion of thyroxinelike I^{127} , found equally in thyroid of adult salmon and of *Protopterus*, is distinctly less than that determined by Wolff and Chaikoff¹²⁴ in a certain number of vertebrate species. This seems to indicate that thyroglobulin does not have a constant composition of iodinated amino acids in all the series of vertebrates as is commonly believed.¹²⁵

the animals were maintained at the same temperature and fasting, shows a considerable reduction of thyroid activity during estivation.¹²⁹

The I^{131} uptake, which is considerable in the active animal, as it reaches 86 per cent 72 hours after injection, is no more than 1 to 2 per cent in the animal in estivation (FIGURE 11). The study of the distribution of I^{131} fixed in the thyroid indicates a slowing of the organic binding and of the synthesis of T_3 and T_4 in the animal in estivation. However, the proportions of T_3 and T_4 in the active animal are always very low (1 to 3 per cent). In the active animal the labeled thyroid hormones T_3 and T_4 appear in the plasma in small quantities 24 hours after the injection and in more significant quantities in 3 days, but they cannot be found in the animal in estivation (FIGURE 11).

The content of I^{127} in the thyroid is about 4 times more elevated in encysted animals. On the other hand, the thyroxinelike fraction, which is from 10 to

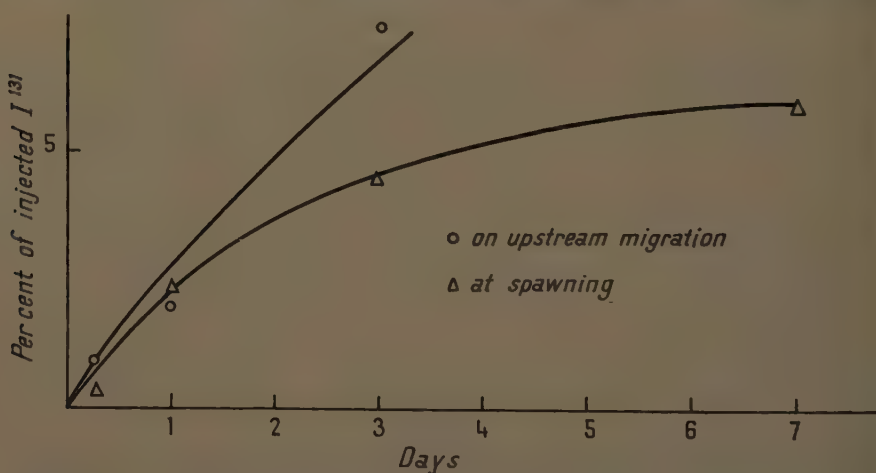


FIGURE 10. Uptake of I^{131} by thyroid of a adult salmon in upstream migration and at spawning.

12 per cent of thyroïdal I^{127} , does not seem to be modified, but the PBI considerably decreases and represents only 10 per cent of the circulating iodine, instead of the 27 to 39 per cent in the active animal (FIGURE 12).

These results seem to indicate that estivation involves in the lungfish a hypophysectomylike state, at least concerning the pituitary secretion of TSH. The fact that the content of pituitary TSH is little different in the active animal and in estivation¹⁷ does not contradict this hypothesis, since the pituitary content does not determine the quantity of hormone released in the blood.

METAMORPHOSIS IN AMPHIBIA

Although Money *et al.*⁴⁶ were unable to show in tadpoles of *R. pipiens* a difference between I^{131} turnover during metamorphosis and during nonmetamorphosis, the majority of authors observe a correlation between the metabolism of I^{131} and the different phases of metamorphosis. In *R. clamitans* the thyroid uptake 3 to 4 hours after the injection of I^{131} is 0.07 and 0.12 per cent

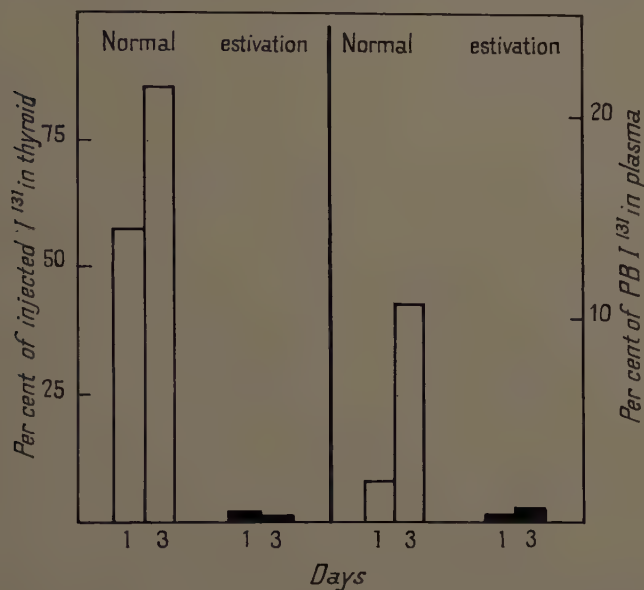


FIGURE 11. Influence of estivation in *Protopterus* on the uptake of I^{131} by thyroid and percentage of PBI^{131} in the plasma.

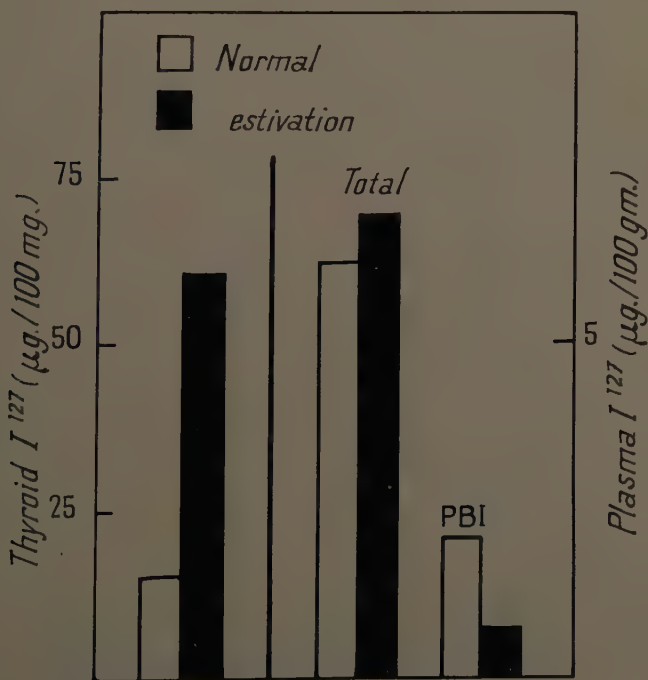


FIGURE 12. Influence of estivation in *Protopterus* on thyroid and plasma iodine (I^{127}).

when the length of the hindlimbs is about 3 mm.; it is from 0.23 to 0.49 per cent when the hindlimbs are to 13 to 14 mm., and reaches its maximum, 0.9 to 2.4 per cent, in animals in advanced stages of metamorphosis (imminent or actual forelimb emergence).¹⁰⁶

The species most studied has been *X. laevis*. It is interesting to compare the data of Dodd¹³⁰ on the thyroid uptake of I^{131} with these of Saxén *et al.*,^{63,131} who utilize 6 parameters of thyroid and pituitary function (percentage of colloid and of epithelium in the thyroid, fixation of I^{131} in the thyroid, PBI^{131} and PBI^{127} in extrathyroidal tissues, size of the nuclei of the para-aminosalicylic acid-positive (PAS-positive) cells in the anterior pituitary), and with the recent chromatographic data obtained by Shellabarger and Brown.⁹² All these experiments are done by immersing tadpoles in a solution of I^{131} for 48 or 96 hours before sacrifice. The following outline can be made:

(1) In premetamorphic states and at the beginning of metamorphosis the I^{131} uptake is low, thyroidal I^{131} is nearly all in the inorganic form, and MIT and DIT represent only 3 to 4 per cent. The percentage of colloid is high, that of the epithelium relatively small, and the size of the nuclei of PAS-positive cells is reduced. PBI^{131} is relatively increased and PBI^{127} is low. The activity of the thyroid is thus reduced.

(2) In the course of the growth of the hindlimb, until the eruption of the forelimbs (the metamorphic climax), the uptake of I^{131} increases considerably, the maximum being reached at the climax. Correspondingly, T_4 appears in significant quantities (on the average 4 per cent of thyroidal I^{131} with traces of T_3). Iodide represents not more than 49 to 67 per cent, and MIT and DIT increased to 28 to 49 per cent. PBI^{127} and PBI^{131} are not modified, the percentage of epithelium increases, and the colloid decreases. Finally, the nuclear size and the granulation of the PAS-positive cells of the anterior pituitary increase. These facts indicate an increase of thyroid activity and an augmentation in hormone synthesis, but the liberation of hormone does not seem to be increased.

(3) The eruption of the forelimbs is associated with abrupt change in the activity of the anterior pituitary and the thyroid. The thyroidal I^{131} decreases, as does the PBI^{131} , while the PBI^{127} of the tissues increases considerably. The proportion of T_4 decreases and T_3 disappears, that of MIT and DIT increases to 77 per cent, and the iodide falls to 20 per cent. Simultaneously, the thyroid follicles collapse, the percentage of epithelium increases, and that of the colloid decreases. The nuclei of the PAS-positive cells and the granulation of the cytoplasm begin to decrease. These facts can be interpreted as indicating a release of thyroid hormone under the influence of TSH, accompanied by an increase in the utilization of this hormone.

(4) In the metamorphosed animal, the I^{131} uptake is small, but the proportion of T_4 attains 10 per cent, that of iodides not being more than 8 per cent, and of MIT and DIT, 79 per cent. The percentage of epithelium decreases, that of the colloid increases, and the PBI^{127} and the PBI^{131} vary little. The size of the nuclei of the PAS-positive cells decreases. These facts seem to indicate a decrease of the release of TSH, involving a moderate activity of the thyroid gland.

It is therefore possible to conclude with Saxén *et al.*^{63,131} that "normal metamorphosis is dependent on a steady increase in the level of thyroid hormone in the tissues or, in other words, on increased anterior pituitary and thyroid activity."

Quantitative Measure of the Thyroid Secretion of Lower Vertebrates

Quantitative estimation of the thyroid secretion alone permits a knowledge of the functional capacity of the gland. Thus far the only results published concerning the lower vertebrates concern the newt.¹³² This lack of data, contrasting with numerous facts brought forth concerning mammals and birds, is probably explained by the fact that the most-utilized method for such a determination, substitution therapy (administration of a quantity of T_4 that will stop the development of a goiter in an animal treated with antithyroid drugs,¹³³ or the liberation of labeled thyroid hormone¹³⁴) is difficult to apply to lower vertebrates. On the one hand, the goitrogenic action of antithyroid drugs is often weak, slow to appear, and variably effective in poikilotherms,^{87,135,136} and thyroid weight cannot be evaluated in cyclostomes and teleosts. On the other hand, the release of labeled thyroid hormone is often very slow, particularly at low temperatures. Genest and Adams¹³² overcame the difficulty by evaluating "the minimum amount of thyroxine necessary to induce molting and to prevent the usual cessation of molting and maintain a normal skin in the thyroidectomized newt." The secretion of the male *Triturus viridescens* evaluated by this method is from 0.024 to 0.046 μg , of T_4 , or 0.78 to 1.50 μg . of hormonal iodine/100 gm. of body weight, the average weight of newts being 2 gm. (Adams, personal communication). This elevated secretion, almost that of the rat, should be considered a maximum because the method of replacement therapy gives figures that are too high,¹³⁷ and the needs of the newt for thyroid hormone are probably highest at the time of molting.

We have calculated, from curves of disappearance of plasma, I^{131} , from the thyroid uptake of I^{131} , and from the content of inorganic I^{127} in the plasma,* the average value of the thyroid secretion of several species of lower vertebrates

* The rate of secretion of thyroid hormone (H) is estimated from the equation (unpublished data):

$$H = \frac{(k_1 + k_2)U_{\max}It}{I_0}$$

where, according to Stanbury *et al.*,¹²² k_1 = rate constant of thyroid accumulation of iodide, k_2 = rate constant of excretion of iodide, U_{\max} = theoretical uptake: fraction of labeled iodide entering thyroid, I = inorganic I^{127} per gram of plasma, t = time in hours, and I_0 = I^{131} per gram of plasma extrapolated at time 0.

Total disposal rate ($k_1 + k_2$) is calculated from the curve of plasma radioactivity.

U_{\max} is calculated by the equation:

$$U_{\max} = \frac{U_t}{1 - e^{-(k_1 + k_2)t}}$$

in which U_t represents organic I^{131} in the thyroid at time t .

This calculation gives the quantity of I^{127} organically bound in the gland during a unit time, and eliminates the interference of the iodine leaving the thyroid (Nadler and Leblond¹³⁸) in the first intervals after injection of I^{131} . It is supposed that in any animal in a steady state the hormonal iodine secreted in the blood is equal to that organically bound by the gland.

and, for some of these species, the ecologic and ethologic variations (TABLE 12). An examination of TABLE 12 permits the following remarks to be made.

(1) The secretion is generally less than that measured in homeotherms except for the *Protopterus*, in which it is higher than that of the teleosts and near that of the newt. This fact shows a new resemblance, in addition to the anatomic and ontogenic resemblances between the lungfish and the Amphibia.

(2) In teleosts there is considerable variation from one species to another, and there seems to exist a relationship between the activity of the species con-

TABLE 12
RATE OF SECRETION OF THYROID HORMONE IN LOWER VERTEBRATES

Species	Conditions		Rate of secretion (μ g. hormonal iodine /100 gm. body weight/day)	Time turn- over (days)
	Stage	Temp. (°C.)		
Lamprey (<i>Petromyzon marinus</i>)	Female, upstream migration	14	0.005	75
Carp (<i>Cyprinus carpio</i>)	Adult	16	0.0002	4500
Eel (<i>Anguilla anguilla</i>)	Silver, normal	25	0.040	78
	Silver, normal	6.5	0.014	416
Trout (<i>Salmo gairdnerii</i>)	Adult	17	0.175	—
	Immature, quiet water	10	0.180	—
	Immature, upstream swimming	10	0.35	—
Trout (<i>Salmo fario</i>)	Immature	14	0.030	358
Salmon (<i>Salmo salar</i>)	Parr	14	0.058	172
	Silvered parr	14	0.084	138
	Smolt, migrating	14	0.140	38
	Adult, upstream migration	9-14	0.12	—
	Adult at spawning	7-10	0.033	—
Lungfish (<i>Protopterus annectens</i>)	Active	23	0.81	5.7
	Estivation	23	0.015	687
Salamander (<i>Triturus viridescens</i>)	Adult male		0.78-1.50*	

* Data from Genest and Adams.¹³²

sidered and the secretion of T_4 . It is thus that the Salmonidae have a secretion greater than that of the eel, itself higher than that of the carp, which is sluggish. Similarly, the rainbow trout secretes more hormone in stream water than in quiet water.

(3) The secretion of T_4 varies with the temperature. It is more elevated in the eel at 24° C. than at 6° C. As has been pointed out, the secretion in the lungfish is high at 25° C.

(4) The physiological condition of the animal affects the secretion. It is for this reason that the secretion of T_4 by the lungfish is considerably decreased during estivation, although it is maintained at the same temperature. Simi-

larly, the secretion by salmon varies significantly in the course of its migratory cycle. The secretion is elevated during the migratory states: smolt in catadromous migration, salmon in anadromous migration. It is distinctly lower in nonmigratory states: parr, and mature salmon on the spawning ground. Finally, the secretion increases distinctly in the parr during its development into the smolt.

(5) The relatively elevated values observed in the rainbow trout are probably partially due to foods that are iodine-rich, since these trout originate in hatcheries. It is known that the T_4 secreted increases in the rat when the iodine content of the diet increases.^{138,139}

The only data on the biological half life of thyroidal iodine in fishes were contributed by Fromm and Reineke,¹⁴⁰ who measured *in vivo* the uptake and output of I^{131} by the thyroid of rainbow trout. They found the biological half life to be 15.5 days but, as pointed out by these authors, it is not a true figure because this calculation is based on values that are influenced by both thyroidal and nonthyroidal I^{131} output rates; the actual output half life is more than 15.5 days. In our study, the turnover time (thyroidal I^{127} per 100 gm. body weight/the rate of secretion per day) varies generally in the same way as the secretion. It is particularly low in the active *Protopterus* and considerable in the carp (TABLE 12).

The more elevated thyroid secretion in the active species and in the trout in stream, which consume more oxygen, the increase of the secretion in the silvered parr and the smolt, corresponding with an increase in oxygen consumption,¹⁴¹ and the decrease in the secretion of the lungfish in estivation, corresponding with a decrease in oxygen consumption,¹⁴² all seem to indicate that, in lower vertebrates, contrary to classic beliefs, the thyroid may be implicated in respiratory exchanges.

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CONCENTRATION AND ORGANIC BINDING OF RADIOIODINE BY THE THYROID GLAND

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This paper reviews some aspects of the concentration and organic binding of radioiodine by the thyroid gland. In particular, a summary is given of some information obtained from autoradiography and from the analysis of the kinetics of the accumulation of radioiodine by the thyroid gland. This presentation illustrates the general thesis that, although autoradiographic studies indicate that the thyroid gland is heterogeneous and therefore complex, certain aspects of the kinetics of its radioiodine metabolism appear to be simple.

Autoradiographic Localization of Radioiodide

Radioiodide is localized in follicles of the thyroid gland in animals in which organic binding is blocked by a single dose of a goitrogen.¹ The concentration of radioiodide in the epithelial cells seems to be much less than in the lumen of the follicle,² since the autoradiographic density above tangential sections of follicles (sections containing only cells) was much less than that over colloid.² Moreover, the concentration of radioiodide in the lumen of large follicles seems the same or occasionally larger than in small follicles,² depending upon the species of animal and the physiological state of the thyroid. Since in small follicles the cells are a larger fraction of the follicle than in larger follicles, these results indicate that the ratio of the radioiodide concentration in the whole follicle to the serum radioiodide concentration (F/S) is less for small follicles than for large follicles in the same thyroid gland.

Autoradiographic Localization of Protein-Bound Radioiodine

As indicated by autoradiographic studies, protein-bound radioiodine seems to be localized almost entirely in the lumen of the follicle.^{3,4} At short time intervals after injection the autoradiographic density or the concentration of protein-bound radioiodine seems to be higher in the smaller follicles than in the larger ones. By quantitative autoradiography and by the use of a method of reconstructing the population of follicles from sections,⁵ Nadler *et al.*⁶ found that the dependence of the autoradiographic density on follicle size is what might be expected if the uptake of radioiodine were proportional to the surface area of the follicle. The greater autoradiographic density above the smaller follicles than above the larger ones is due to the larger surface-to-volume ratio of the smaller follicles.

Autoradiographs at short time intervals after injection (or if the colloid is very viscous) indicate that protein-bound radioiodine is localized almost entirely in the colloid at the periphery of the lumen.^{3,4,7} The autoradiographic rings are frequently of nonuniform density^{3,4,7} and indicate that in many cases not all the cells of a follicle are clearing radioiodine from the blood at the same rate.

The observation that, at 10 or 15 sec. after intravenous injection of radioiodide, protein-bound I^{131} is found only in the lumen of the follicle⁴ indicates either (1) that the iodination of thyroglobulin occurs in the cell and that secretion of the protein-bound radioiodine is exceedingly rapid, or (2) that the iodination occurs in the lumen of the follicle at the periphery of the colloid. The obviously crucial experiment to see if colloid uncontaminated by cells can form organic iodine from iodide apparently has not been done.

Kinetics

Although the thyroid gland is heterogeneous, it seems possible that some aspects of radioiodine metabolism might be superficially simple; for example, certain aspects of the behavior of the thyroid gland might be represented faithfully by the behavior of an average follicle. To understand the kinetics of radioiodine metabolism of the thyroid gland we have ignored, as a first approximation, the complexities of nonuniform follicle size and function and

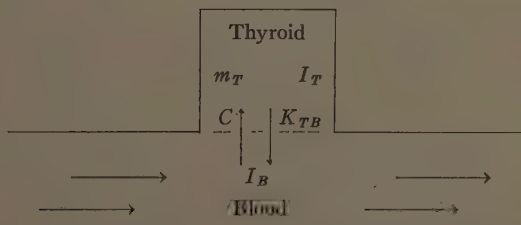


FIGURE 1. Open 2-compartment model: organic binding blocked. Reproduced by permission from *The American Journal of Physiology*.⁸

considered the thyroid gland to be made up of an aggregate of duplicate follicles. To simplify the analysis of the problem, attention was restricted to as simple an experimental situation as possible. The thyroid gland was considered an *in vivo* perfusion system; hence, concentrations of I^{131} in the thyroid were related to blood levels of radioiodide rather than to dose.

Organic Binding Blocked

Consider first the case in which organic binding of radioiodine is blocked by a single dose of propylthiouracil injected an hour before radioiodide injection.⁸ In the simplest model (FIGURE 1) the thyroid gland is considered to be a well-mixed box, equivalent to a population of uniform follicles. The thyroid gland is of mass m and has a thyroid radioiodide concentration I_T . Radioiodide is transferred into the thyroid gland from the blood at a rate equal to the radioiodide content of C ml. of serum per minute. C is called the one-way clearance. Radioiodide is also transferred out of the thyroid and into the blood. Each minute a fraction K_{TB} of the radioiodide in the thyroid gland is transferred to the blood. K_{TB} is called the exit-rate constant. The constant values of C and K_{TB} are a consequence of a steady state with respect to stable iodide.

An important relation derived from the simple model is that in the steady state, if the blood radioiodide concentration is constant

$$T/S = \frac{C/m}{K_{TB}} \quad (1)$$

where T/S is the ratio of the radioiodide concentration in the thyroid gland to that in the serum. Since the T/S is a ratio of rate constants, it may be expected to be an unsatisfactory measure of thyroid activity, for any stimu-

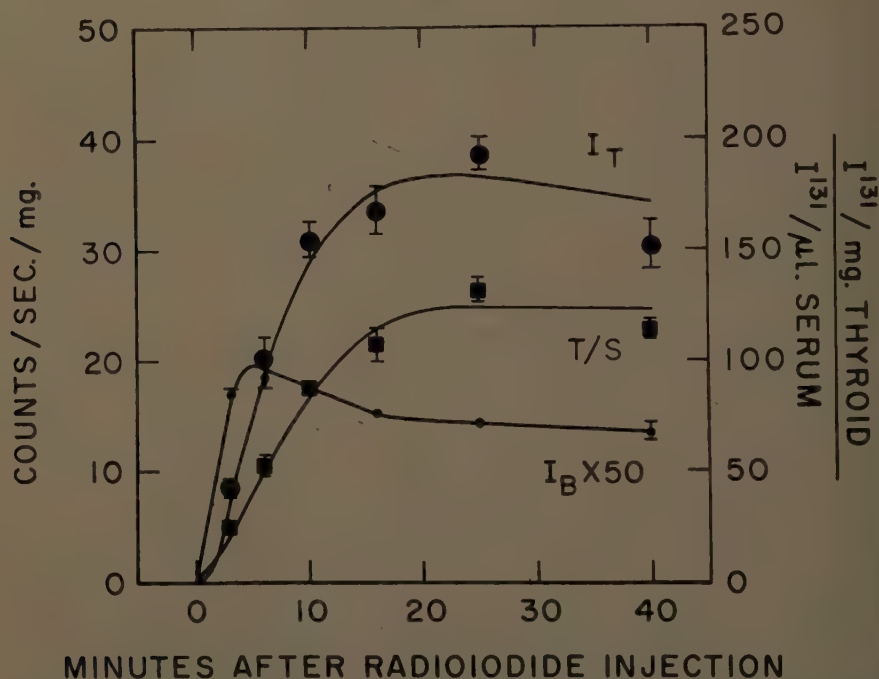


FIGURE 2. Equilibration curve: rats fed stock diet. The smooth curve through the serum radioiodide concentration points (I_B) is assumed. The curves for the thyroid radioiodide concentration (I_T) and for the T/S were calculated from I_B , using the open 2-compartment model in FIGURE 1. Reproduced by permission from *The American Journal of Physiology*.⁸

lus that changes both C/m and K_{TB} proportionately leaves the T/S unchanged. It appears that changes in the exit-rate constant are responsible for some puzzling properties of the T/S .⁸

A typical set of experimental data obtained in rats fed a stock diet is shown in FIGURE 2. The smooth curve through the small solid circles is assumed to be the true serum radioiodide concentration as a function of the time. The smooth curves through the thyroid radioiodide data and through the T/S data were calculated from the blood data. The smooth theoretical curves fit the data within experimental error. The values for the clearance and exit-rate constant that gave good fits of the model with experimental data for the stock diet and for other experimental conditions are summarized

in TABLE 1. The one-way clearance varied markedly with the physiological state of the gland. Hypophysectomy decreased the one-way clearance. Stimulation of the gland by a low-iodine diet or by thiouracil feeding yielded larger one-way clearances than a high-iodine diet. Exit-rate constants were fairly constant, independent of the level of iodine in the diet. However, hypophysectomy decreased the exit-rate constant, whereas thiouracil feeding increased the exit-rate constant.

Part of the reason for the large exit-rate constant in animals fed thiouracil may be simply that the follicles in the thyroids from these animals are abnormally small. It can be shown that small follicles would be expected to have a larger exit-rate constant than large follicles. From EQUATION (1)

$$K_{TB} = \frac{C/m}{T/S} \quad (2)$$

Since the one-way clearance as well as the effective clearance might be ex-

TABLE 1
CONSTANTS CHARACTERIZING THE EQUILIBRATION OF RADIOIODIDE
BETWEEN THE THYROID GLAND AND BLOOD⁸

Group	Mice		Rats	
	Clearance per mg. thyroid, C/m $\mu\text{l./mg./min.}$	Exit-rate constant, K_{TB} min.^{-1}	C/m $\mu\text{l./mg./min.}$	K_{TB} min.^{-1}
Hypophysectomized	2.4	0.025	1.51	0.050
Purina	4.7	0.050	3.2	0.11
Stock diet	9.0	0.041	15.0	0.12
Pabulum	15.2	0.055	19.0	0.10
Thiouracil-fed	41.0	0.33	30.0	0.23

pected to vary proportionally to the surface area of the follicle,⁶ C/m would be expected to vary inversely as the diameter. Furthermore, the radioiodide concentration in the lumen of follicles and hence the F/S or the T/S generally seemed to be relatively independent of the follicle size.² In this case, K_{TB} would be expected to vary as C/m , that is, inversely as follicle diameter. In the special case of very small follicles the F/S for the follicle would be expected to be abnormally low. Very small follicles therefore may be expected to have an abnormally high exit-rate constant. Considering the large range in follicle sizes in a thyroid gland and therefore the large range of exit-rate constants, it is surprising that a single exit-rate constant fits the data so well.

Since thiocyanate administration decreases the T/S , we have examined the effect of thiocyanate on the one-way clearance and exit-rate constants (TABLE 2). It was found that the one-way clearance was essentially independent of the thiocyanate level in the serum. The entire effect of thiocyanate in decreasing the T/S was due to an elevation of the exit-rate constant. The interpretation of this finding is uncertain as yet. A more complex model, which is a somewhat better representation of the distribution of radioiodide

in the thyroid gland than the simple model, yields the same empirical equation. However, the interpretation of the exit-rate constant differs in the two models. In the complex model (FIGURE 3), each duplicate follicle of the thyroid gland consists of cells and lumen, and the radioiodide is largely localized in the lumen. Transfer of radioiodide from the thyroid gland to the blood is still determined by a one-way clearance, C ml. of serum being emptied of radioiodide per minute. On the other hand, there are three other rate constants describing the transfer of radioiodide from the compartment with the first

TABLE 2
EFFECT OF THIOCYANATE ON ONE-WAY CLEARANCE AND EXIT-RATE CONSTANTS

Species	Serum thiocyanate conc. mg./100 ml.	C/m $\mu\text{l.}/\text{mg.}/\text{min.}$	K_{TB} min.^{-1}
Mouse	0	9.0	0.041
	1.96	10.0	0.27
Rat	0	15.0	0.12
	1.33	16.0	0.37

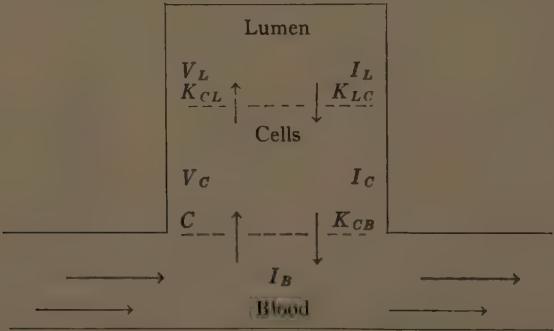


FIGURE 3. Open 3-compartment model: organic binding blocked. Reproduced by permission from *The American Journal of Physiology*.⁸

subscript to the compartment with the second subscript. The effective exit-rate constant is given⁸ by

$$K_{TB} = \frac{K_{LC}K_{CB}}{K_{CL}} \tag{3}$$

Any process that decreases the concentration of radioiodide in the lumen relative to the cell, for example, by increasing K_{LC} or decreasing K_{CL} , will yield an increased effective exit-rate constant. In the present state of our knowledge thiocyanate could affect any one of the three transfer constants. An interpretation of the thiocyanate effect by our methods will require the determination of the effect of thiocyanate on each of these transfer constants individually.

Organic Binding Permitted

A simple model for the thyroid gland in which organic binding of radioiodine is permitted can be constructed (FIGURE 4). In this model, radioiodide is transferred from blood to the thyroid gland at a rate equal to the radioiodine content of C ml. blood/min. Radioiodide in the thyroid gland is disposed of in two ways. A constant fraction of it equal to K_b (the binding-rate constant) is incorporated into organic iodine each minute. Another fraction K_{TB} (an exit-rate constant) is transferred from the thyroid radioiodide pool back into the blood each minute. This model is useful only at rather short time intervals after injection of radioiodide, since direct transfer of organic iodine from the thyroid gland to the blood and deiodination of thyroidal organic iodine, with return of the liberated radioiodide to the radioiodide pool of the thyroid gland, are neglected. In this paper discussion of

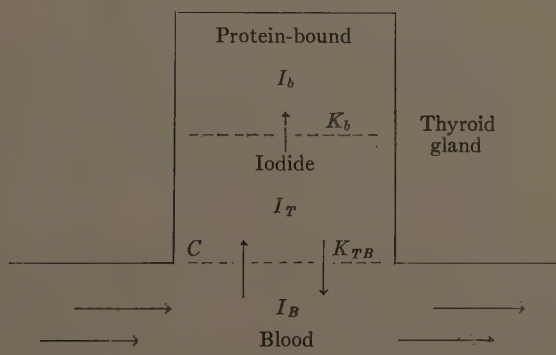


FIGURE 4. Open 3-compartment model: organic binding permitted.

this model is limited to attempts to estimate binding-rate constants from experimental data.

To compute the binding-rate constants, the thyroid radioiodide concentration must be known. This is generally small compared with the protein-bound radioiodine concentration. Since the protein-bound I^{131} is slightly unstable, it would appear desirable to have some objective criterion by which to determine the magnitude of the contribution to thyroid radioiodide from thyroidal protein-bound I^{131} . In the absence of such a criterion our approach has been to work at such short time intervals after injection that the PB I^{131} is not too large compared with the thyroid radioiodide concentration, so that the decomposition of thyroid protein-bound iodine can be neglected. A typical set of experimental data for rats fed Purina Lab Chow is shown in FIGURE 5. The curve marked I_T through the experimental points (*solid circles*) was assumed to be the thyroid radioiodide concentration as a function of the time. The curve I_b is a theoretical curve for protein-bound I^{131} calculated from the thyroid radioiodide concentration data using the model in FIGURE 4. It fits the experimental data within experimental error. Typical values of K_b for various groups of animals are shown in TABLE 3. Binding-

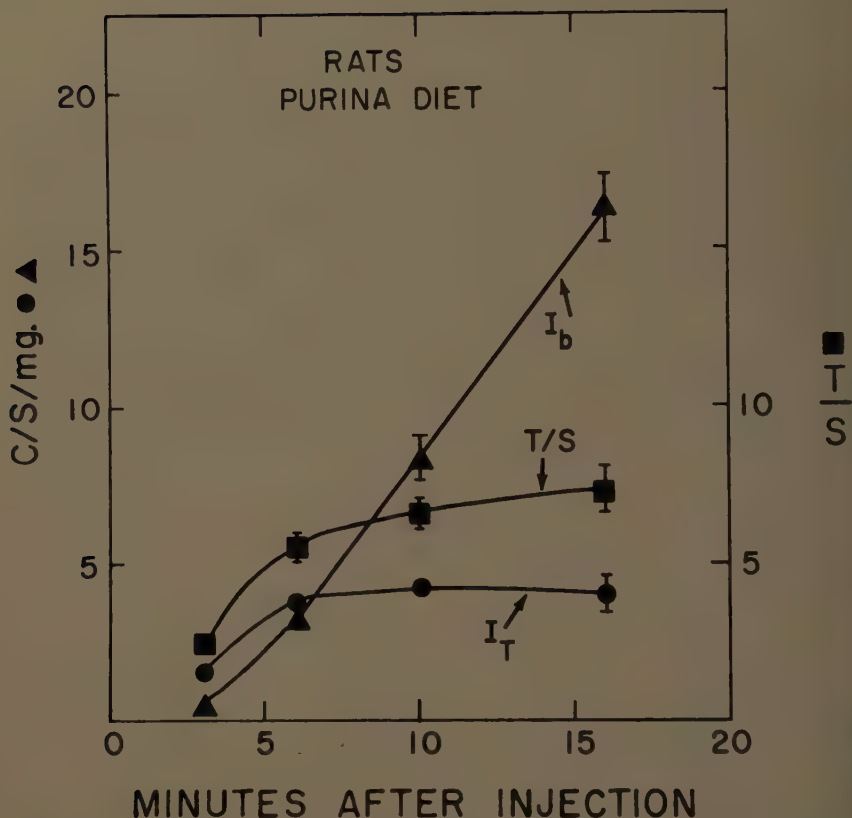


FIGURE 5. Radioiodine uptake curve: rats fed Purina Lab Chow. The ordinate on the left is counts/sec./mg. The smooth curve through the thyroid radioiodide concentration points (I_T) is assumed. The smooth curve through the thyroidal protein-bound radioiodine points (I_b) was calculated from I_T , using the 3-compartment model of FIGURE 4.

TABLE 3
BINDING-RATE CONSTANTS
(Fraction per Minute)

Group	Mice	Rats
Hypophysectomized	0.09-0.01	0.10
Purina	0.15	0.32
Stock diet	0.86	1.70
Pablum	1.20	1.15

TABLE 4
STEADY-STATE VALUES OF THE T/S IN RATS

Group	Organic binding blocked	Organic binding permitted
Hypophysectomized	25	7.3
Purina	31	7.5
Stock diet	115	5.7
Pablum	187	11.5

rate constants as high as 1.9 have been observed; that is, the amount of I^{131} bound per min. is almost twice the amount of radioiodide present. Such high binding-rate constants are not equivalent to instantaneous binding. Even with a binding-rate constant of 1, 10 per cent of the I^{131} in the gland will be expected to be in the form of iodide even as late as 10 min. after injection. Hypophysectomy has a strong effect on the binding-rate constant, especially in mice. In hypophysectomized mice, instead of being constant, K_b decreases with time after injection. Analysis indicates that the data might be explained if the thyroid gland had 2 populations of follicles, one with a high binding-rate constant, low radioiodide concentration, and rapid equilibration, and the other with low binding-rate constant, high concentration of radioiodide, and slow equilibration.

Comparison of the T/S with Binding Permitted and Binding Blocked

The most reliable data on values of the T/S thus far are in the rat (TABLE 4). The T/S was much less in animals in which binding was permitted than in animals in which binding was blocked by a single dose of propylthiouracil. The major cause of the low T/S in animals in which binding is permitted seems to be the rapid removal of concentrated radioiodide from the radioiodide pool by the binding process.

Conclusion

Although the thyroid gland is a heterogeneous aggregate of follicles, over limited time intervals (shortly after the injection of radioiodide) some aspects of thyroidal radioiodine metabolism appear to be simple. Accumulation of radioiodine by thyroid glands can be described quantitatively by the simplest of models. The values of simply interpretable constants can be determined from experimental data, and they yield interesting insights into some aspects of thyroid physiology.

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SOME FACTORS THAT AFFECT THYROID HORMONE SYNTHESIS

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In the normal thyroid gland, hormone synthesis occurs in three stages: concentration of iodide from the blood, iodination of tyrosine yielding mono- and diiodotyrosine, and conversion of these to thyroxine and 3,5,3'-triiodothyronine. These iodo amino acids are stored in thyroglobulin; after hydrolysis by the thyroid protease, thyroxine and triiodothyronine are secreted into the blood. In normal conditions, the iodotyrosines do not leave the thyroid; they are deiodinated by the thyroid deiodinase, and the iodide thus obtained becomes available for reutilization in the thyroid hormone biosynthetic cycle.

The anterior pituitary secretion thyrotrophin (TSH) is of paramount importance in the control of all stages of thyroid hormone production. This, and the role of the central nervous system in regulating TSH secretion are discussed by other contributors to this publication and will not be considered here.

Adrenal Gland

The effects of adrenal cortical hormones and of adrenocorticotrophic hormone (ACTH) on thyroid function have been studied by numerous workers (see Money¹). Large doses of cortisone depress the thyroid, as do desoxycorticosterone acetate, ACTH, and various forms of stress. Cortisone administration is followed by increased renal excretion of iodide; Money¹ concludes that its depressant action on the thyroid is due to this enhanced competition of the kidney for iodine.

Ingbar and Freinkel² have re-evaluated the evidence concerning the effects of ACTH and cortisone on thyroid function; they conclude that, while the short term effects may in part be due to increased renal clearance of iodide, the principal effects result from the suppression of TSH secretion by the pituitary.

The effects of the medullary hormone adrenaline on the thyroid are thought to be mediated via the anterior pituitary and adrenal cortex by stimulating increased TSH and ACTH secretion.¹ Adrenaline may also act by increasing the peripheral utilization of thyroid hormones.³ Adrenaline decreases thyroid blood flow in the cat, rabbit,⁴ and man;⁵ noradrenaline increases it in man, but has no effect in the dog.⁵ Söderberg⁴ has shown that the decreased thyroidal blood flow in the rabbit, induced by either adrenaline or pitressin, was accompanied by a parallel fall in thyroidal I¹³¹ uptake. When an increased blood flow was produced by intravenous injection of bromolysergic acid diethylamide, a corresponding rise in thyroidal I¹³¹ uptake was observed. Urinary excretion of I¹³¹-labeled endogenous or exogenous thyroid hormone is increased after injection of both adrenaline and noradrenaline in the rat,⁶ rabbit,⁴ and dog.⁵

Ackerman and Arons⁷ have shown that, after intravenous infusion of adrenaline and noradrenaline in the dog pretreated with I^{131} , the PBI^{131} levels in blood from the thyroid vein rose from 1.5 to 17 times the control values within 15 to 95 min. after the infusion. The rate of blood flow through the thyroid was not measured in these experiments. The effect of adrenaline was demonstrable in hypophysectomized animals and was therefore a direct effect on the thyroid. Preliminary chromatographic studies showed that the rise in blood PBI^{131} was due to increases in both thyroxine and triiodothyronine.

Mowbray and Peart⁵ have been unable to detect any changes in thyroidal uptake of I^{131} or circulating PBI^{131} levels after infusions of adrenaline and noradrenaline in man.

Species Differences

It is recognized that certain animals, such as the rat and the mouse, have very active thyroids compared with those of the guinea pig, rabbit, and chick. Wollman and Wodinsky⁸ have shown by autoradiographic techniques that the thyroid of the mouse contains I^{131} -labeled colloid within 11 sec. of intravenous injection of tracer doses of I^{131} iodide. Galton and Pitt-Rivers (*see* Pitt-Rivers⁹) have recently studied the rate of I^{131} organification in the thyroids of different species. Groups of animals were injected intravenously with I^{131} under light anesthesia, and their thyroids were removed and analyzed for organic I^{131} and I^{131} iodide by the method of Pitt-Rivers *et al.*¹⁰ at intervals from 15 sec. to 60 min. after the injection. In the rat, mouse, and hamster, incorporation of I^{131} into thyroglobulin (PBI^{131}) was exceedingly rapid, reaching values of more than 50 per cent of the total thyroidal I^{131} in 0.5 to 2 min. In the rhesus monkey, 50 per cent of thyroidal I^{131} was bound 5 min. after the injection, but in the guinea pig and rabbit 50 per cent organic binding was reached only after about 30 min. These authors have also found that organic binding of I^{131} is slow in the chick thyroid and, as might be expected, is slower in the gland of the day-old bird than that of the 7-day-old bird. It must be remembered that the fraction of iodine that is protein-bound at any time will depend on the efficiency of the iodide-trapping mechanism.

Iodide

The direct relationship between dietary iodine deficiency and the incidence of goiter has been debated since the work of Chatin (*see* Harington¹¹) more than 100 years ago. Recently Kelly and Snedden¹² have reviewed work on endemic goiter all over the world. Most authors agree that the primary cause of goiter is iodine deficiency.

The effect of excessive iodide on the thyroid varies in different species. It is marked in birds; Wheeler and Hoffmann¹³ have shown that addition of 0 to 64 mg. of iodide/kg. to the diet of hens caused an increase in thyroid weight from 98 mg. to 330 mg. In 10-day-old chicks from the birds, thyroid weights increased from 3.0 mg. to 12.6 mg. These authors¹⁴ also produced goitrous chicks by injecting iodide into eggs on the sixteenth day of incubation, but the effect was not so striking as in the chicks from iodide-fed hens.

The rat is very resistant to the goitrogenic effect of iodide. Wolff and Chaikoff¹⁵ showed that a single dose of 500 μg . I^{127} (labeled with I^{131}) inhibited thyroidal organic binding of iodine, but that the effect was transient and had disappeared within 24 hours. Furthermore,¹⁶ the blocking effect of iodide could not be maintained even with a sustained level of plasma iodide. These findings were confirmed by Galton and Pitt-Rivers,¹⁷ who also showed that a high dietary level for 7 weeks did not inhibit iodine binding.

Recently Pitt-Rivers (unpublished) has tried to produce goiter in rats by prolonged iodide feeding. Two groups of 50-gm. male rats were given (1) 100 mg. I^{127} (as KI) per 100 ml. drinking water for 28 weeks; (2) 400 mg. I^{127}

TABLE 1
EFFECT OF PROLONGED IODIDE TREATMENT ON I^{131} BINDING IN RAT THYROID

Time after I^{131} (hours)	PBI^{131} (%)			
	Controls	Acute I^{127}	Low I^{127}	High I^{127}
1	91	4	78	47
2	98	4	83	65
6	98	—	93	87
16	98	97	98	98

TABLE 2
EFFECT OF PROLONGED IODIDE TREATMENT ON THYROIDAL I^{131} MIT/DIT IN RAT THYROID

Time after I^{131} (hours)	I^{131} MIT/DIT	
	Controls	Treated
1	0.9	1.3
2	0.9	1.1
6	—	0.7
16	0.6	0.6

per 100 ml. drinking water for 23 weeks. The controls received tap water. During the first 2 months of the experiment, there was no change in the BMR of the treated animals compared with that of the controls. At the end of the experiment, the animals were injected intraperitoneally with I^{131} and killed at different time intervals after the dose. The thyroid glands were removed, weighed, and analyzed for protein-bound I^{131} (PBI^{131}) and iodide I^{131} . Neither the body weights nor the thyroid weights differed from those of the controls. The rate of I^{131} binding in both groups of animals is shown in TABLE 1. It can be seen that the high dose of iodide, equivalent to about 4 mg. per day, had some effect on the rate of I^{131} binding, but the low dose of iodide (about 1 mg. per day) had very little effect. The elevated monoiodotyrosine:diiodotyrosine (MIT/DIT) ratios found by Galton and Pitt-Rivers¹⁷, at early intervals after a single dose of 500 μg . I^{127} were not observed in the chronically iodide-treated rats (TABLE 2).

Raben³¹ has shown that the inhibitory action of iodide on I^{131} binding can be reversed by simultaneous administration of thiocyanate. Rats maintained on a low iodine diet were injected with 600 μ g. iodide labeled with I^{131} . Another group was injected with 600 μ g. iodide and 7.5 μ g. thiocyanate; in the latter group, the amount of thyroidal iodine bound after 6½ hours was 3 times as great as in the group that was not given thiocyanate. The effect of 30 mg. thiocyanate on the blocking action of 100 μ g. iodide was less marked.

Rall and Pitt-Rivers (unpublished) have found that perchlorate will also reverse the effect of excessive iodide in the rat thyroid. Groups of rats were injected intraperitoneally with 3 doses of I^{127} : 50 μ g., 150 μ g., and 500 μ g. labeled with 50 μ c. I^{131} . One hour later, one-half the animals were injected intraperitoneally with 20 mg. perchlorate. After another hour, all the animals were bled, killed, and their thyroids were analyzed for PBI¹³¹ and I^{131} iodide. The I^{131} in the serum was also determined. The results are shown in TABLE 3.

TABLE 3
EFFECT OF PERCHLORATE ON INHIBITION BY IODIDE OF THYROIDAL IODINE BINDING

Treatment	Thyroid μ g. I^{127}		Iodide T/S	Thyroid PBI %
	PBI	Iodide		
50 μ g. I	0.23	0.19	31.0	55.0
50 + ClO_4^-	0.051	0.016	1.8	76.0
150 μ g. I	0.009	1.12	51.0	0.6
150 + ClO_4^-	0.015	0.016	0.4	43.0
500 μ g. I	0.011	1.14	25.0	1.0
150 + ClO_4^-	0.045	0.042	0.7	52.0

These data indicate that perchlorate reversed the inhibitory action of iodide. The absence of a perchlorate effect in the lowest I^{127} group is probably due to the fact 50 μ g. I^{127} had only a slight effect on iodine organification. From Raben's³¹ and these results it appears that iodide inhibition can be reversed by drugs that lower the thyroid/serum iodide levels (T/S). This is compatible with Serif and Kirkwood's¹⁸ hypothesis concerning the mechanism of iodide inhibition of iodination in the thyroid.

Pitt-Rivers (unpublished) has also studied the effects of chronic and acute iodide administration in the young guinea pig and of acute iodide treatment in the 7-day-old chick. In the acute experiments all the animals were injected intraperitoneally with 500 μ g. I^{127} + I^{131} , and the thyroid glands were analyzed for PBI¹³¹ and I^{131} MIT/DIT. In the chronic experiments, young male guinea pigs were given 10 mg. I^{127} (as KI) per 100 ml. in their drinking water for 28 weeks. The results are shown in TABLES 4 and 5. These data show that the acute effects of iodide on PBI¹³¹ binding in the guinea pig and chick are less pronounced than in the rat. Also, the high I^{131} MIT/DIT ratios observed by Galton and Pitt-Rivers¹⁷ soon after a single dose of I^{127} in the rat were not found in the other species.

In the chronically I^{127} -treated guinea pig, some thyroid enlargement was

observed; the glands weighed about 40 per cent more than those of the controls. Moreover, at early time intervals after I^{131} injection organic binding was considerably inhibited, and I^{131} MIT/DIT was somewhat elevated 2 and 6 hours after the dose.

The histology of the thyroid glands of rats and guinea pigs after prolonged iodide treatment will be described by Janet S. F. Niven elsewhere.

The sensitivity of the human thyroid to excessive iodide is probably in-

TABLE 4

ACUTE EFFECT OF IODIDE ON I^{131} BINDING IN THE GUINEA PIG AND CHICK THYROID

Time after $I^{127} + I^{131}$ (hours)	PBI ¹³¹ (%)			
	Guinea pig		Chick	
	Controls	Treated	Controls	Treated
1	79		71	36
2	88	12		53
4		44		
6	91		99	88
8		74		
16		83	99	98

TABLE 5

ACUTE EFFECT OF IODIDE ON THYROIDAL I^{131} MIT/DIT
IN GUINEA PIG AND CHICK THYROID

Time after injection (hours)	I^{131} MIT/DIT			
	Guinea pig		Chick	
	Controls	Treated	Controls	Treated
1	1.3		1.1	1.8
2	1.0	2.9		1.4
4		1.2		
6	0.9		0.9	1.1
8		1.0		
16		0.9	0.80	0.9

intermediate between that of the rat and guinea pig. Several reports have appeared of goiter in subjects after prolonged iodide therapy,¹⁹ although it is likely that there are many humans whose thyroids have not been depressed by iodide. Paley *et al.*²⁰ have studied a patient who developed a goiter after several years of iodide treatment and found that, shortly after medication was withdrawn, most of a tracer dose of I^{131} could be discharged from the thyroid by thiocyanate 24 hours after the dose. The ability of the gland to organify I^{131} was restored several months after iodide medication had ended.

Several reports have appeared in the literature on goiter in humans resulting from the ingestion of iodopyrine, an iodinated derivative of phenazone (1,5-dimethyl-2-phenyl-3-pyrazolone). Morgans and Trotter²¹ have dis-

cussed the cause of iodopyrine goitrogenicity and have concluded that it is probably due to the iodide liberated from the molecule, since phenazone is inactive. Brownstone and Pitt-Rivers²² produced evidence in support of this idea, by showing that iodopyrine and iodide in equivalent doses in rats produced almost identical responses in terms of inhibition of thyroidal I^{131} binding, while phenazone had no effect.

Antithyroid Drugs

Thiocyanate and perchlorate. Some years ago Barker²³ described 2 patients who developed goiters after treatment of hypertension with thiocyanate. Since then, the goitrogenic action of thiocyanate in man²⁴ and experimental animals has been amply demonstrated.²⁵ Thiocyanate goiter can be prevented by simultaneous administration of iodide²⁶ and the drug has been shown to act by inhibiting iodide concentration by the gland,²⁷⁻³⁰ although high doses will also inhibit iodide binding.³¹ Iodide already concentrated by the thyroid is discharged by thiocyanate.³⁰

Thiocyanate is not concentrated by the thyroid gland,³²⁻³⁴ but enters it by simple diffusion. It is metabolized in the thyroid to give chiefly sulfate,^{33,34} which is also the principal metabolite of thiourea, thiouracil, and methyl thiouracil. Maloof and Soodak³⁴ have postulated an interesting theory that thiocyanate may be involved in a thyroid homeostatic mechanism, since it is formed in the body during the detoxification of cyanide. Moreover, they showed that there was only a small difference between the amount of thiocyanate normally present in rat serum³⁵ and the concentration in the serum after a dose (20 μ moles) that produced maximal inhibition of the thyroidal iodide trap.

Freinkel and Ingbar³⁶ have shown that anoxia or cold reversibly inhibit the iodide-concentrating mechanism of the thyroid in surviving sheep thyroid slices, blocked with 1-methyl-2-mercaptoimidazole. They have also shown³⁷ that iodide transport *in vitro* is depressed by compounds that depress respiration (cyanide, arsenite, azide) and by others, including dinitrophenol, that dissociate phosphorylation from oxidation.

Few studies have been made on the mechanism of inhibition of the thyroidal iodide trap by thiocyanate. It has been suggested that thiocyanate acts by competing with iodide for the trapping site. However Berson and Yalow³⁸ have shown that a more likely mechanism is an inactivation or temporary poisoning of the trap. This is supported by their quantitative data on the rate of discharge of I^{131} from the glands of human subjects by graded doses of thiocyanate and by the observation that a constant dose of thiocyanate can discharge varying amounts of carrier iodide I^{127} added to the tracer dose.

Wollman and Reed³⁹ have studied the effects of PTU and thiocyanate on the rate of entry (one-way clearance) of iodide from the blood to the thyroid and on the reverse process (exit-rate constant) in rats and mice. They have shown that the one-way clearance is greatly increased in both species by PTU, but is unaffected by thiocyanate; the exit-rate constant is increased by both PTU and thiocyanate. A new concept of an open three-compartment thyroid model has been invoked to explain the unequal distribution of iodide

between the cells and lumen of the follicle. Since thiocyanate had no effect on the one-way clearance but increased the exit-rate constant, and in accordance with other data discussed by the authors, it could act by inhibiting the transport of iodide from the cell to the lumen, though other mechanisms were not excluded.

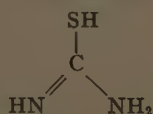
Kanaya⁴⁰ has recently shown that thiocyanate increases oxygen consumption in beef thyroid slices, but decreases the rate of oxidative phosphorylation at concentrations above 10^{-4} M; I^{131} uptake was also depressed by the same range of thiocyanate concentrations. Kanaya inferred that thiocyanate depressed the amount of energy produced by high-energy phosphate bonds that are required for the active transport of iodide into the thyroid.

Other anions, of which perchlorate is the most active,⁴¹ also interfere with the thyroidal iodide trap in the rat and in man, and perchlorate has been used^{42,43} in the treatment of hyperthyroidism. As already stated, large doses of thiocyanate inhibit organic binding of iodine as well as thyroidal iodide concentration,³¹ but binding of I^{131} in the rat thyroid has been shown to lead to a normal distribution of iodinated amino acids when iodide concentration is partially⁴⁴ or completely¹⁰ inhibited by perchlorate.

Anbar *et al.*⁴⁵ have demonstrated inhibition of I^{131} uptake in propylthiouracil-blocked rat thyroids by monofluorosulfonate, difluorophosphate, and fluoroborate ions. These ions have the same volume and charge as perchlorate. The authors conclude that the thyroidal iodide-trapping mechanism cannot distinguish between ions with the same volume and charge, but will concentrate them irrespective of their chemical nature. Experiments with isotopically labeled ions in this series would help to test this hypothesis.

Sulfonamides and thioureas. In 1943, MacKenzie and MacKenzie⁴⁶ and Astwood *et al.*²⁶ showed that certain sulfonamides, notably sulfapyridine and sulfadiazine and some thiourea derivatives produced goiters in the rat, mouse, and dog. Sulfaguanidine also produced a fall in the basal metabolic rate of the rat, but had no antithyroid action in the guinea pig and chick.⁴⁶ Sulfanilamide inhibited I^{131} binding by surviving sheep and beef thyroid slices.⁴⁷

Thiouracil and its methyl and propyl derivatives possess powerful antithyroid properties, as have other drugs containing the thiocarbamide grouping,



such as the mercaptoimidazoles and amino thiazoles. Many of the goitrogenic drugs contain sulfur, but this is not essential for activity: *p*-aminobenzoic acid in high dosage is goitrogenic in the rat⁴⁸ and inhibits thyroid hormone biosynthesis *in vitro*;⁴⁹ 3-amino-1,2,4-triazole is a powerful goitrogen in the rat;⁵⁰ resorcinol, butazolidine, *p*-aminosalicylic acid, and cobalt have all been shown to produce goiter in man.¹⁹

It has been suggested²⁵ that thiourea and related compounds inhibit organic iodine binding in the thyroid owing to the fact that the SH grouping in the molecule reacts with iodine and reduces it to iodide. In the light of present evidence, this explanation can be no more than a partial one. First, many

compounds that do not contain a potential SH group possess thiourealike activity. Second, if the above mechanism were true, it should be possible to overcome the antithyroid action of the thiocarbamides by iodide; this has not been demonstrated. Third, it has been shown that many antithyroid drugs inhibit oxidizing enzyme systems. Astwood⁵¹ has reviewed evidence for the various hypotheses concerning the mechanism of antithyroid action and has concluded that inhibition of the thyroid peroxidase(s) affords the most likely explanation.

It has been shown⁹ that respiratory inhibitors and anaerobiosis inhibit organic binding of iodine by surviving thyroid tissue. However, Suzuki⁵² believes that organic binding of iodine is related to the rate of oxidative phosphorylation rather than to oxygen consumption for the following reasons: compounds that depressed uptake of P^{32} (methylthiouracil, 2,4-dinitrophenol) by thyroid slices also depressed I^{131} uptake. Methylthiouracil had no effect on oxygen consumption, and dinitrophenol stimulated it; succinate also stimulated oxygen consumption, but was without effect on P^{32} and I^{131} uptake.

Richards and Ingbar⁴⁴ have studied the effects of graded doses of propylthiouracil (PTU) on I^{131} metabolism in rat thyroids. Rats were injected with 0.01 to 3.2 mg. PTU 30 min. before and 90 min. after I^{131} and killed 4 hours after I^{131} . Analysis of their thyroids showed that labeled MIT/DIT rose from 0.62 in the controls to 36.8 in the group receiving the highest dose of PTU. Further, thyroxine (T_4) formation was inhibited. Slingerland *et al.*⁵³ have reported similar findings; they fed diets containing 0.01 to 0.0001 per cent PTU to groups of rats for 4 days and killed the animals 7 hours after a tracer dose of I^{131} . Thyroidal MIT/DIT ratios rose from 0.21 in the controls to 7.3 in the animals on the highest dose of PTU. When the animals were killed 24 hours after I^{131} injection and withdrawal of goitrogen, elevation of I^{131} MIT/DIT and T_3/T_4 was also observed. Richards and Ingbar⁵³ detected T_3 in only 50 per cent of their controls and in none of their experimental animals; this would be expected at the time interval (4 hours) at which they removed the thyroids after the I^{131} dose.

Pitt-Rivers (unpublished) has investigated the iodinated compounds in the thyroids of rats made goitrous with aminotriazole, sulfapyridine, and sulfadiazine. Three groups of 100-gm. male rats were fed 1 per cent of these drugs in their diets for 24 to 32 days. They were then injected intraperitoneally with 100 μ c. I^{131} and killed 2 hours later. Their thyroids were removed, weighed, and analyzed for PBI^{131} and iodide I^{131} . PBI^{131} was hydrolyzed and analyzed for iodinated amino acids. The results are shown in TABLE 6.

All 3 compounds were powerfully goitrogenic, as previously reported.^{26,46,48,50} Aminotriazole almost completely inhibited I^{131} binding in the thyroids, but the effects with the sulfa drugs were variable. In 1 group of rats on sulfapyridine 66 per cent of I^{131} was bound 2 hours after injection, and the I^{131} MIT/DIT was normal. In the other sulfapyridine group and in both the sulfadiazine groups, there was considerably less PBI^{131} , and I^{131} MIT/DIT was greatly elevated. Slingerland *et al.*⁵³ have shown that, in the rat, treatment for 4 days with 2.5 per cent dietary sulfanilamide produced elevated thyroidal I^{131} MIT/DIT ratios when the animals were killed 7 hours after

I^{131} injection. However, when the animals were killed 24 hours after I^{131} injection and withdrawal of the sulfanilamide diet, the thyroidal I^{131} MIT/DIT values were normal. This probably explains the high PBI^{131} and I^{131} MIT/DIT values found in one group of sulfapyridine-fed rats (TABLE 6), since the interval between the ingestion of food and I^{131} injection was not known.

A further study has been made on the effect of perchlorate on the acute inhibition of iodine binding by PTU and other goitrogenic compounds (Pitt-

TABLE 6
EFFECT OF PROLONGED GOITROGEN TREATMENT ON THYROIDAL PBI^{131}
AND I^{131} MIT/DIT IN THE RAT

Treatment	Days	Thyroid wt. per 100 gm. body wt.	PBI^{131} (%)	I^{131} MIT/DIT
None	—	8.3	98.0	0.85
Aminotriazole	24	46.0	0.3	—
Sulfapyridine	30	42.8	66.0	0.8
	31	41.0	22.2	12.0
Sulfadiazine	31	65.7	12.6	11.5
	32	61.8	8.8	5.0

TABLE 7
EFFECT OF PERCHLORATE ON THE ACUTE INHIBITION OF THYROIDAL IODINE
BINDING BY VARIOUS GOITROGENIC SUBSTANCES

Compound	Dose (mg.)	No. of animals	Percentage of dose in thyroid		
			Total	Bound	PBI^{131} (%)
None	—	5	0.570	0.445	78.1
Propylthiouracil	5	5	0.153	0.00087	0.57
Propylthiouracil + perchlorate	20	5	0.0052	0.00041	7.9
Propylthiouracil	10	5	0.194	0.00021	0.11
Propylthiouracil + perchlorate	20	5	0.0078	0.00023	2.9
Aminotriazole	50	4	0.105	0.00047	0.45
Aminotriazole + perchlorate	20	4	0.0095	0.00045	4.7
Sulfapyridine	50	5	0.24	0.023	9.6
Sulfapyridine + perchlorate	20	8	0.028	0.012	41.3
Sulfadiazine	50	4	0.22	0.011	5.0
Sulfadiazine + perchlorate	20	8	0.030	0.013	44.3

Rivers, unpublished). Groups of Sprague-Dawley 150-gm. male rats were injected intraperitoneally with PTU, aminotriazole, sulfapyridine, and sulfadiazine; 45 min. later I^{131} was injected and the animals were killed after another hour. In each group a subgroup was injected with perchlorate 45 min. after I^{131} and 1 hour before being killed. The thyroid glands were immediately removed and counted in a well-type scintillation counter for total I^{131} . After chromatographic separation of protein-bound iodine and iodide, PBI^{131} was determined. The results are shown in TABLE 7.

From these data, it is seen that the amount of PBI^{131} in the glands of the

animals given goitrogen alone was of the same order as that in the glands of animals given goitrogen plus perchlorate. With 50-mg. doses of sulfapyridine and sulfadiazine, a significant amount of I^{131} was protein-bound and, even with 10 mg. PTU, inhibition of binding was not complete. Perchlorate did not influence the inhibitory action of these drugs: the nondischargeable I^{131} was already there; however, the minute amount present was not detected by the less sensitive technique used by Pitt-Rivers *et al.*¹⁰ It should be pointed out that the percentages of PBI^{131} found by these authors in the thyroids of rats pretreated with thiouracil before PTU and perchlorate injections were sometimes considerably higher (up to 60 per cent) than the percentages of PBI^{131} obtained with single doses of PTU followed by perchlorate, as shown in TABLE 7. This might be due to differences in treatment, strain of rats, or dietary or other unknown factors.

The concentration of iodide by the thyroid is not inhibited by chronic administration of sulfonamides.³⁰ Their goitrogenic activities appear to result from the suppression of thyroid hormone synthesis rather than from the almost total inhibition of iodine organification obtainable with the thiouracils. As in the case of PTU,^{44,53} monoiodotyrosine formation seems to be less inhibited by sulfonamides than is diiodotyrosine formation. The essential toughness of the mechanism responsible for monoiodotyrosine synthesis toward drugs^{10,44,53} or of cellular damage⁹ is emphasized again.

An alternative hypothesis has been propounded,⁵⁴ namely, that these drugs do not act on a specific diiodotyrosine enzyme, but that the amount of diiodotyrosine formed is limited by the concentration of iodine in the thyroid relative to the amount of tyrosine available for iodination. Although Slingerland *et al.*⁵³ showed that the administration of iodide together with sulfanilamide did not alter the MIT/DIT ratio, this observation still leaves open a choice between the two views, since the amount of iodide administered would be expected by itself to give rise to an elevated MIT/DIT ratio. It is also possible that diiodotyrosine formation is not a simple function of the concentration of iodine in the gland, but may be influenced both by this factor and by the toxic effects of drugs on the enzyme system involved.

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IODOPROTEINS IN THE THYROID

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Introduction

The thyroid gland contains a protein first studied so long ago that it has been noted only recently that little is really known of its structure. This protein is thyroglobulin, which comprises most of the iodine of the thyroid. Recently other iodoproteins in the thyroid present in smaller amounts have been partially characterized. We shall present some new data on the old protein and review our work on the nonthyroglobulin iodoproteins.

Thyroglobulin

Preparation. Since thyroglobulin is so abundant in the normal thyroid of the large mammals studied, little work has been done on its purification. A crude aqueous extract of a thyroid mince is ~ 70 per cent thyroglobulin. Derrien and his co-workers pointed out the unusually sharp range of insolubility of thyroglobulin in ammonium sulfate or potassium phosphate buffers.¹ Hence salting out has been used generally as a method of purification. However, relatively little purification is actually achieved by this procedure even when repeated several times, and substantial losses were incurred. It might be well to mention what thyroglobulin is: in the hog it is a globular protein with $S_{20,w}^0 \cong 19.2$ to 19.4 that contains variable amounts of iodinated amino acids.²⁻⁴ The sedimentation rate is emphasized since the determination of this physical constant readily differentiates it from other thyroid proteins. For some of the studies to be reported it was desirable to achieve a preparation of thyroglobulin almost entirely free from the slower- and faster-sedimenting components that contaminate thyroglobulin solutions prepared by the salting-out methods of Derrien^{1,3} (FIGURE 1). A method of differential centrifugation was used in which saline extracts of thyroid tissue were sedimented and the lighter proteins removed. Three or 4 sedimentations in this manner (No. 40 rotor, 40,000 rpm, 260 min.) removed essentially all the lighter components. A final centrifugation to remove the heavier protein ($S_{20,w} \cong 25$) afforded a preparation, as illustrated in FIGURE 1*b*, that showed a highly symmetrical boundary in the ultracentrifuge with very little if any slower or faster sedimenting material.

Molecular Properties

The size and shape of thyroglobulin at neutrality. Various studies of the molecular properties of hog thyroglobulin are in quite good agreement with each other and give a molecular weight close to two-thirds million. The sedimentation and diffusion data may be interpreted in terms of a model form, that is, a prolate ellipsoid of revolution, in which case it would have an axial ratio of about 8.2^3 . Our experiments on calf thyroglobulin at neutral *pH* values indicate rather close conformity in molecular parameters with those of hog

thyroglobulin. However, at least in one respect the behavior of the calf thyroglobulin molecule differs substantially from that of the hog. Calf thyroglobulin forms a new, slower sedimenting component at pH values somewhat above neutrality, and the concentration of this component increases as the pH is raised. Above pH 9.5 two additional boundaries are formed in the ultracentrifuge. Heidelberger and Pedersen examined the pH stability of hog thyroglobulin and found it to be stable as high as pH 11.3.² At pH 12, however, S-19 thyroglobulin was no longer evident and was replaced by 2 new boundaries having sedimentation rates of 12.4 and 9.2S.



FIGURE 1. Ultracentrifuge patterns of (A) phosphate-purified thyroglobulin at 0.5 per cent (*upper*) and 1.5 per cent (*lower*); (B) ultracentrifugally prepared thyroglobulin.

The Dissociation of Thyroglobulin into Subunits

At pH 9.5. Lundgren and Williams observed the formation of a new sedimenting component when solutions of hog thyroglobulin were dialyzed to eliminate free salt.⁵ Since the concentration of this component, which they called α protein, increased with increase in protein concentration, they rejected a dissociative reaction and assumed that it represented an isomeric form of thyroglobulin. The sedimentation rate of this new component was markedly dependent on protein concentration and had a value of about 10 to 11 Svedberg units when extrapolated to infinite dilution. On the addition of salt to dialyzed thyroglobulin solutions, the sedimentation diagram returned to that found before dialysis, indicating a reversible, ionic strength-dependent equilibrium between thyroglobulin and α protein.

The observations on the α protein by Lundgren and Williams⁵ were carried out in solutions devoid of neutral salt. Since the sedimentation properties of charged macromolecules depend on the ionic strength of the medium,⁶ a critical

evaluation of the properties of the α protein of hog thyroglobulin becomes quite difficult. However, we have observed the formation of a component that resembles α protein in its sedimentation behavior in solutions of calf thyroglobulin in 0.10 *M* KNO₃.⁷ The relative amount of this component could be increased to 25 to 30 per cent by reducing the KNO₃ concentration to 10⁻³ *M* at neutrality or by raising the *pH* to ~ 9.5 in 0.10 *M* KNO₃.

The sedimentation constant of this new component was 12.1S at zero protein concentration (for convenience we will refer to this component as 12-S and native thyroglobulin as 19-S). In the further characterization of this slower-sedimenting particle it became apparent that its properties did not conform to those expected, assuming that it was an isomeric form of thyroglobulin. In that event the diffusion coefficient of a mixture of 19-S and 12-S would be smaller than that of 19-S alone. We found, however, that the diffusion coefficient increased from 2.49 to 2.56×10^{-7} when the *pH* was raised from 6.0 to 9.5.⁷ The concentration of 12-S increased from zero at *pH* 6 to 16 per cent at *pH* 9.5, as determined by analytical ultracentrifugation (see below concerning the low concentration of 12-S at *pH* 9.5 in this experiment).

Further data confirming a dissociation into smaller-sized particles was obtained by light-scattering measurements.⁷ If 12-S were an unfolded form with the same mass as thyroglobulin, no change in turbidity would be expected as 19-S was converted to 12-S. However, a decrease in turbidity was observed when the *pH* was increased. In fact, the rate of formation of 12-S could be measured by the fall in scatter. At *pH* 9.5 the change in scatter was complete in about one half hour. At higher *pH* values somewhat longer periods of time were required.

In detergents. Additional hydrodynamic evidence for the splitting of native thyroglobulin was furnished by its behavior in solutions containing the anionic detergent sodium dodecyl sulfate (SDS).⁸ In 10⁻³ *M* SDS (and 0.10 *M* KNO₃), the sedimentation pattern of thyroglobulin revealed 2 symmetrical boundaries (*S* values of 17.3 and 11.8) in about equal concentrations. Nevertheless this concentration of SDS had no measurable effect on the viscosity. If 12-S were an unfolded isomeric form of thyroglobulin, its frictional ratio (*f/f*₀) would have increased by about the ratio of the sedimentation rates of the 2 components (at zero concentration), that is, 19.4/12.1. A change of this magnitude in 50 per cent of the protein present would lead to an increase in viscosity that could hardly be missed. Since essentially no increase in viscosity occurred, the only feasible conclusion is that 12-S is formed by dissociation of 19-S into 2 equal subunits without much change in asymmetry. Light-scattering data support this hypothesis by showing a decline in turbidity that agrees quantitatively with that expected from a split of 50 per cent of the thyroglobulin into equal halves.

If the 12-S component of calf thyroglobulin is similar in structure to the α protein of Lundgren and Williams,⁵ then it is possible to rationalize their failure to find a decrease in the concentration of 12-S with increasing protein concentration, as required by the law of mass action for a dissociation reaction. In the first place, O'Donnell *et al.* have already pointed out that the Johnston-Ogston effect would lead to an artificial increase in the area of their slower-

moving boundary as the protein concentration was increased.⁴ Second, several of our observations with calf thyroglobulin indicate that the dissociation of S-19 into 12-S molecules at pH 9.5 must indeed be a very slow reaction (at room temperatures) and should therefore possess a significant activation energy. These observations may be summarized as follows: (1) the slopes of the sedimentation versus concentration plots of both 19-S and 12-S were negative; (2) the 2 boundaries were always completely resolved on sedimentation; and (3) the reduced turbidity was independent of protein concentration. In systems where subunits are in rapid equilibrium with each other, as in the case of insulin,⁹ quite a different behavior in all these categories has been reported. It is also not unlikely that the complete elimination of free salt affects the composi-

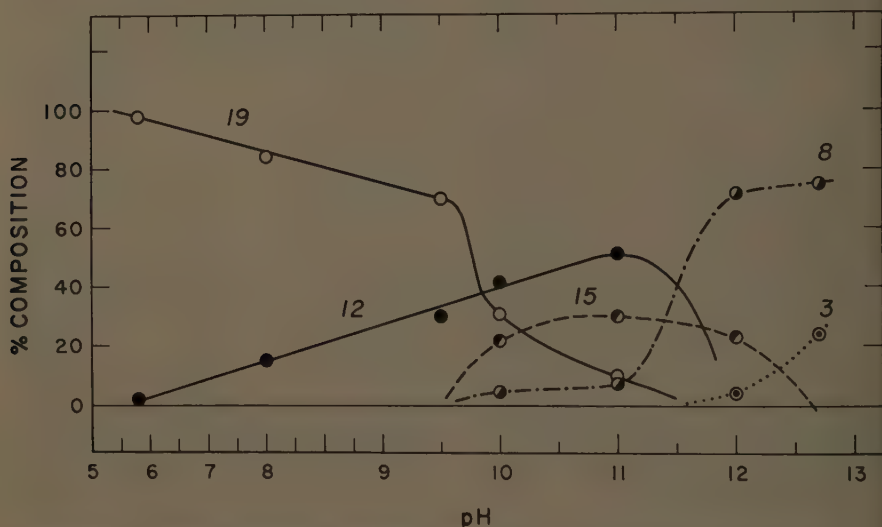


FIGURE 2. Ultracentrifugally determined composition of thyroglobulin at various pHs.

tion of components at different concentrations, since the gegen-ion concentration and hence the ionic strength will vary with protein concentration.

Further Conformational Changes of Thyroglobulin

Effect of higher pH values. The sedimentation pattern of thyroglobulin becomes more complex when the pH is raised above 9.5. Two additional boundaries evidently appear simultaneously, at pH values slightly above 9.5, with sedimentation coefficients of 15 and about 8 and in a weight ratio of about 3 to 1, respectively. The variation in thyroglobulin components with pH observable by ultracentrifugation is summarized in FIGURE 2. The ordinate shows average values obtained on several different preparations of thyroglobulin. Generally, the composition observed at a particular pH varied only ~10 per cent. One recent preparation proved to be much more resistant to alkaline disaggregation in that higher pH values were required to achieve the same degree of breakdown obtained with earlier preparations. This refractory

preparation was used in the diffusion and light-scattering experiments reported in the preceding section, and thus accounts for the low proportion of 12-S in these experiments.* Since the turbidity falls by about 20 per cent between pH 9.5 and 11.2 it seems possible that these 2 new components represent forms of thyroglobulin with about three quarters and one quarter, respectively, of the mass of 19-S.

When the pH of a solution of calf thyroglobulin was raised from neutrality to pH 11, no insoluble protein was formed on subsequent acidification to pH 5.2 with 1.0 *M* acetate buffer even after 3 hours at pH 11. However, at pH 11.6 insoluble protein was formed when the solution was acidified. The denaturation reaction proceeded according to first-order kinetics and showed a

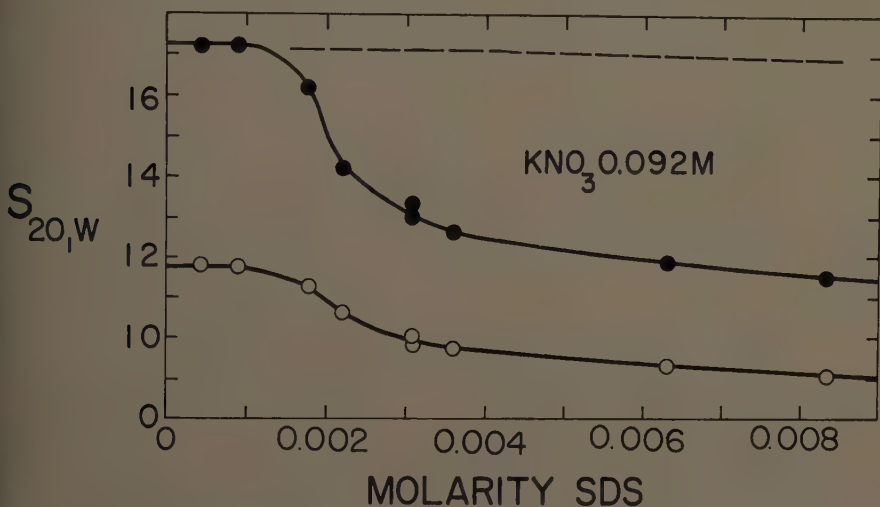


FIGURE 3. Sedimentation coefficient of 19-S and 12-S material as a function of SDS concentration.

very strong pH dependence. About one half the protein was denatured in 5 min. at pH 11.6. At pH 11.9 the half life was about 1 min. Since the ultracentrifuge patterns were largely reversible from neutrality to pH ~ 11 , but were either irreversible or slowly reversible above pH 12, it would appear that the 8-S species, which comprises about 75 per cent of the composition at pH 12, conforms to the definition of a denatured protein. The faster-moving components 12-S and 15-S, on the other hand, are probably globular forms of thyroglobulin in reversible equilibrium with 19-S.

Effect of higher detergent concentrations. Below 0.001 *M* SDS, thyroglobulin dissociates without much change in configuration. Above 0.001 *M* SDS, further dissociation occurs (FIGURE 3), but with concomitant major changes

* It seems possible, although no proof is yet available, that the degree of dissociation at a specific pH could be influenced by the extent of iodination of thyroglobulin. The formation of new boundaries in the ultracentrifuge occurs in the pH region of phenolic hydroxyl ionization, and the pH of these groups is known to be materially affected by iodination of the phenolic ring.¹⁰

in the form of both 19-S and 12-S. Between 0.001 and 0.004 M SDS, the sedimentation coefficient of thyroglobulin and its dissociation product showed a substantial decline that tended to level off at higher concentrations of detergent (FIGURE 3). This transition in frictional properties was observed also in the viscosity, which showed analogous variations with changes in detergent concentration. As illustrated in FIGURE 4, the specific viscosity in 0.01 M KNO_3 increased almost linearly from 0.001 to 0.009 M SDS, whereas in 0.10 M KNO_3 an inflection was observed at about 0.003 M SDS and the viscosity leveled off above $\sim 0.004 M$. The important influence of ionic strength on

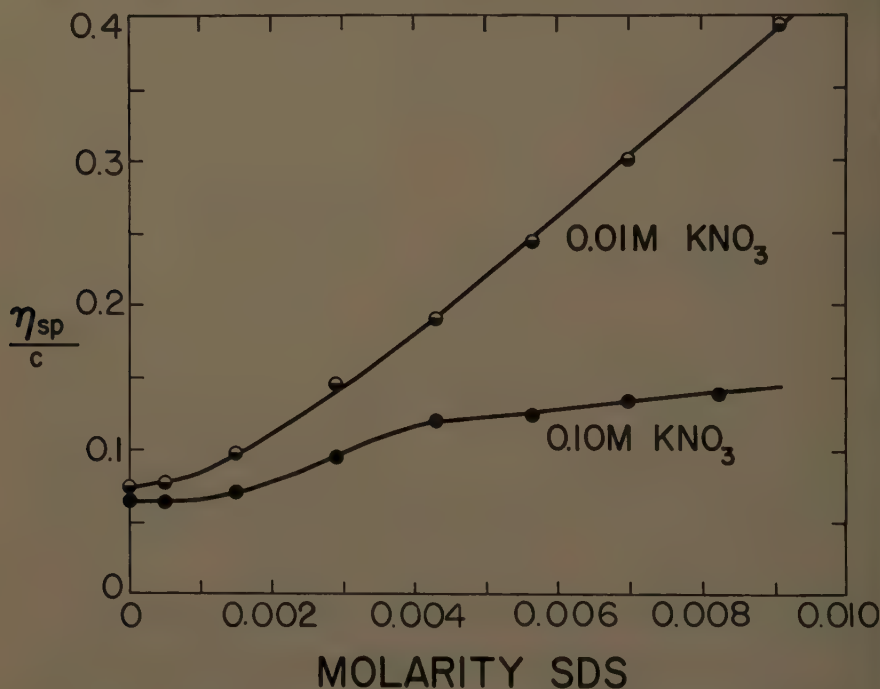


FIGURE 4. Viscosity of a thyroglobulin solution at various concentrations of SDS.

viscosity was seen also in sedimentation. The sedimentation rate of both components 19-S and 12-S in 0.011 M SDS was strikingly dependent on the ionic strength, as shown in FIGURE 5. Results from both types of measurement offer convincing evidence that thyroglobulin and its dissociation product in detergent solutions behave as polyelectrolytes. This implies that the polypeptide chains are more or less free to rotate in solution and conform in their hydrodynamic properties to flexible, randomly kinked coils. The fixed charges due to basic and acidic groups, as well as those due to bound detergent ions, provide a charged backbone to the polypeptide chains. The dimensions of the molecule then will depend primarily on electrostatic interactions. These interactions will be a function of the net charge on the protein and the ionic strength of the medium. Increase in net charge or decrease in ionic concentration will

result in stronger intramolecular repulsions, with a consequent stretching or expansion of the molecular domain of the molecule.

The properties of thyroglobulin tend to revert to those of the native form when the amount of bound detergent is reduced either by dialysis or by dilution. The interaction therefore appears to be largely reversible.

Nonthyroglobulin Iodoproteins

It was believed for many years that thyroglobulin was the only vertebrate protein that was naturally iodinated.¹¹ The first indication that other io-

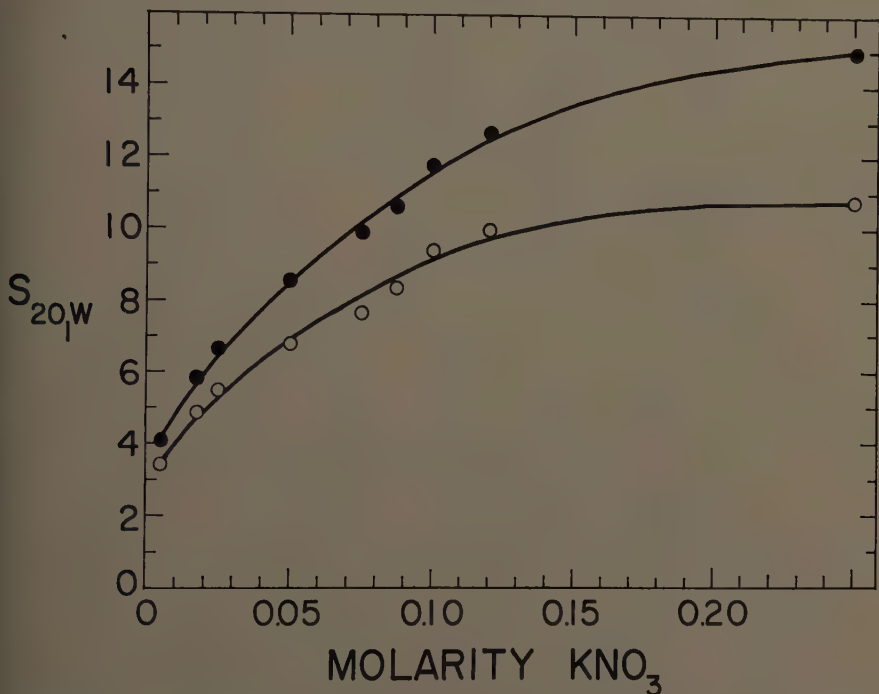


FIGURE 5. The sedimentation coefficient of 19-S and 12-S materials as a function of SDS concentration.

dinated proteins might exist appeared when the sera of certain patients with carcinoma of the thyroid given I^{131} were carefully examined.^{12,13} A material, at first termed compound X, was found that was an iodoprotein with a sedimentation coefficient ($S_{20,w}$) of 4.2S, and an electrophoretic mobility and a salting-out curve similar to that of serum albumin. Other investigators have found nonthyroglobulin iodoproteins in sera of individuals with thyroiditis, congenital goiter, and hypothyroidism.¹⁴⁻¹⁷ In thyroid tissue some differences between iodoproteins from normal and carcinomatous tissue were observed in salting out (in ammonium sulfate) and in electrophoretic mobility, but these appeared to represent variations in the thyroglobulin itself.^{18,19}

Studies of normal thyroid tissue have shown the presence of a slowly sedi-

menting protein containing iodine that has been termed thyralbumin. (Shulman *et al.*,²⁰ and Shulman, personal communication). The relationship of this material to compound X or to the soluble nonthyroglobulin iodoproteins of thyroid tissue described below has not yet been clarified.

We have recently reported a study of a transplantable rat thyroid tumor in which a protein similar to compound X and an insoluble iodoprotein associated

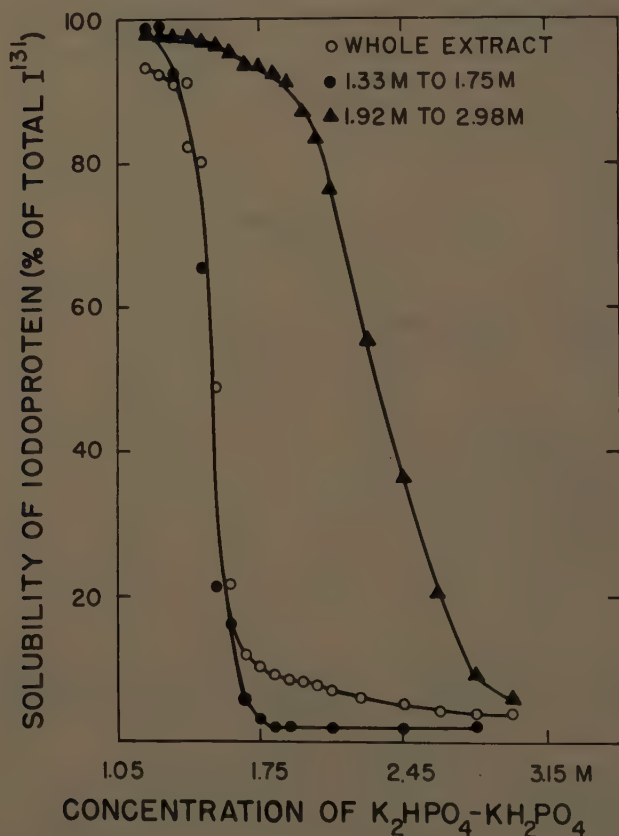


FIGURE 6. Salting-out curves for thyroglobulin and S1 prepared from a rat tumor thyroid extract. Reproduced by permission from *Endocrinology*.²¹

with subcellular particles have been found.²¹ Similar iodoproteins have been found in normal and abnormal thyroid glands of man and sheep.²² In this paper we shall review the salient characteristics of these iodoproteins and present some new data on the insoluble or particulate proteins.

Soluble iodoproteins. Thyroglobulin is easily extracted from thyroid tissue by the simple expedient of freezing and slicing or coarsely grinding the tissue and suspending it for 16 hours or less in 0.9 per cent NaCl. Examination by salting out of such an extract prepared from transplantable rat thyroid tumors after the *in vivo* administration of I^{131} iodide revealed the presence of an iodine-containing component other than thyroglobulin (FIGURE 6). This tumor strain

(Wollman strain 1-8), grown in Fisher rats, was kindly given us by S. H. Wollman and is a highly functional tumor accumulating 30 to 70 per cent of a dose of I^{131} in 24 hours, even with rats fed upon Purina chow, which has a relatively high iodine content.*

S1† can also be differentiated from thyroglobulin by its behavior in the ultracentrifuge, as shown in FIGURE 7. A markedly slower sedimentation rate is evident. In this experiment the solutions were centrifuged at 137,500 g (maximum) in a Spinco No. 40 rotor at 39,000 rpm for 3 hours. At the end of the run the contents of the tube were sampled from the top downward with a needle and syringe. FIGURE 8 shows the analytical ultracentrifuge pattern

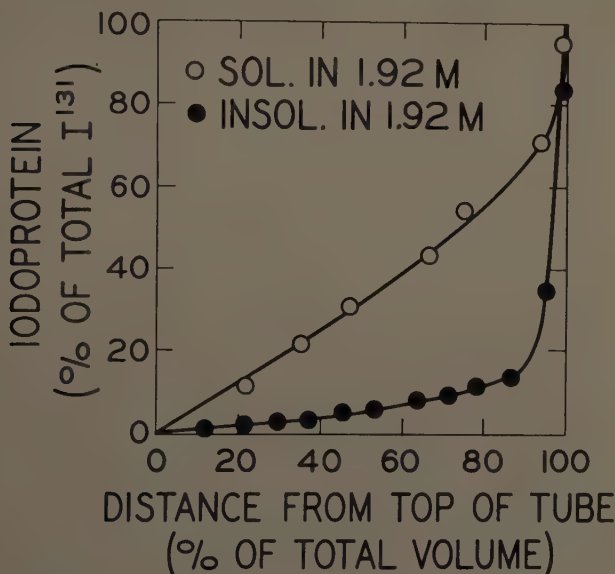


FIGURE 7. Ultracentrifugal behavior of thyroglobulin and S1 obtained from a rat thyroid tumor (B 310). The preparative ultracentrifugation was done at 39,000 rpm for 3 hours in 0.15 M NaCl. Reproduced by permission from *Endocrinology*.²¹

of the phosphate fractionated thyroid proteins; the 1.4 to 1.75 M (PO_4 buffer) fraction is enriched in thyroglobulin ($S_{20} \cong 19$) and the 1.75–2.98 M fraction containing S1 is enriched in $S_{20} \cong 4$ material. The identity of the $S_{20} \cong 4$ material in this figure with the radioiodine-labeled material in FIGURE 7 is, however, only conjectural. The electrophoretic behavior of these phosphate fractions is shown in FIGURE 9. It may be seen that the labeled thyroglobulin travels as a single peak (barbital, pH 8.6) with or without added human serum. S1, on the other hand, shows 3 poorly defined peaks of radioactivity, with or

* The tumor was malignant and was fatal to the rats in 6 to 9 months. These tumors were produced originally by prolonged thiouracil feeding in Fisher rats. At first dependent upon thiouracil treatment of the host for growth, this strain later became autonomous. All the tumors studied and herein reported were derived from a single tumor of the twelfth generation. We have carried this line for an additional 9 generations without significant change in biochemical activity.

† We have defined S1 as a soluble thyroidal iodoprotein identifiable by its unusual solubility in phosphate buffer and its slow sedimentation in the ultracentrifuge.

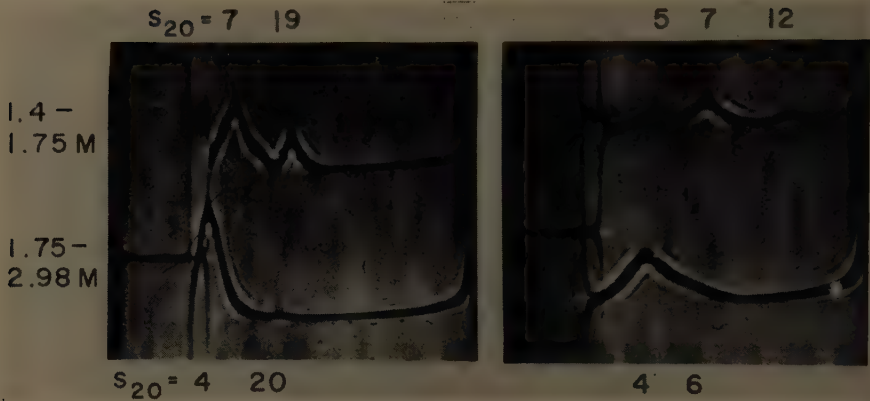


FIGURE 8. Analytical ultracentrifuge patterns of soluble protein fractions from a rat thyroid tumor (B 310). The protein fractions were prepared by precipitation between the limits of $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ buffer indicated. The photograph on the right was taken 28 min. after that on the left. Note the difference in meniscus between the standard and the "wedge" cell. Reproduced by permission from *Endocrinology*.²¹

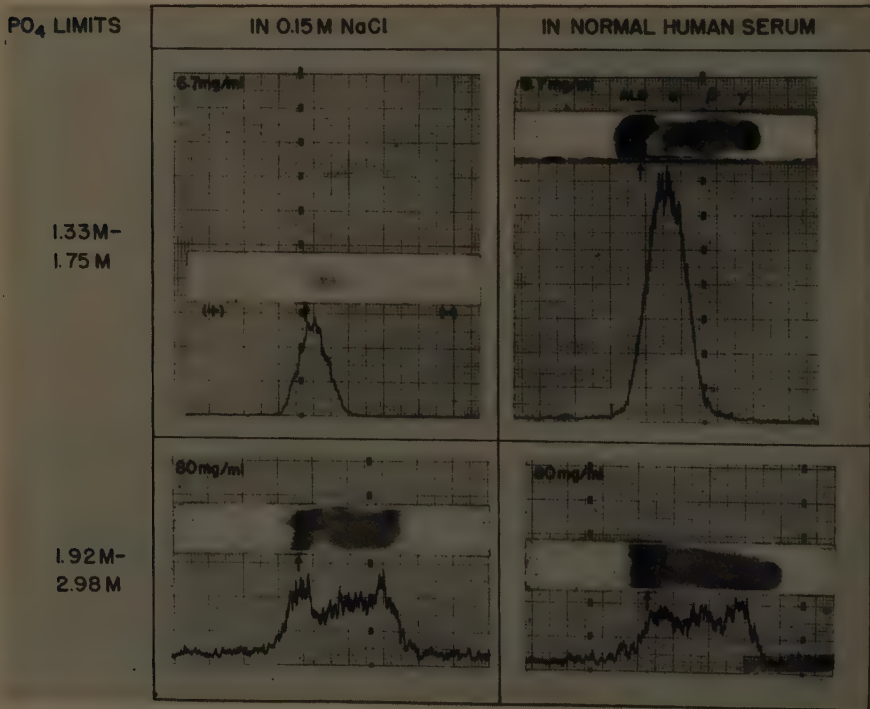


FIGURE 9. Reverse-flow electrophoresis in barbital buffer of soluble iodoproteins of a rat thyroid tumor (B 310).

without added serum. Properly, therefore we should speak of S1-a, S1-b and S1-c, but since we have not isolated and further studied these fractions we shall continue to refer to them as a single protein. The results of hydrolysis of S1

TABLE 1
HYDROLYSIS OF THYROGLOBULIN AND MORE SOLUBLE IODOPROTEINS

Time after I ¹³¹	Insoluble in 1.92 M PO ₄						Soluble in 1.92 M PO ₄					
	O	I	M	D	T ₄	T ₃	O	I	M	D	T ₄	T ₃
1 day	2+	2++	38	29	28	0	14	6	49	31	12	6
7	8	3	31	40	24	0	32	3	54	32	0	0
12	18	11	21	56	20	0	35	11	54	39	0	0

Symbols: O = origin material; I = iodide; M = monoiodotyrosine; D = diiodotyrosine; T₄ = thyroxine; and T₃ = 3'-3,5 triiodothyronine; + = for the origin material the value is per cent of total I¹³¹; and ++ = for all other substances the number listed is per cent of hydrolyzed radioactivity.

TABLE 2
HYDROLYSIS OF TUMOR AND SERUM IODOPROTEIN OF THYROIDECTOMIZED RATS WITH THYROID TUMOR (B 310)

Time after I ¹³¹	Tumor iodoproteins				Serum iodoprotein
	Particulate	Soluble			
		Whole	<1.92 M	>1.92 M	
M/D ratio					
7+ min.	1.4	1.3	0.70	5.4	
30	1.8	1.9	2.1	4.6	
3 hours	1.1	0.86	0.66	2.4	
7	1.0	0.78	0.75	2.1	4.8
1 day	0.74	0.84	1.3	1.9	5.4
2	0.77	0.90			4.6
7	0.57	0.76	0.77	1.7	9.9
12	0.47	0.29	0.38	1.4	
19	0.38	1.0			
M + D/T ₄ + T ₃ ratio					
7 min.	0.38	1.0	0.38	1.4	
30	0.38	47.0	0.38	1.4	
3 hours	14.0	6.3	8.8	1.4	
7	6.3	4.4	2.5	3.0	9.9
1 day	4.7	2.7	2.1	4.9	18.0
2	6.6	2.5			18.0
7	16.0	3.7	3.0	4.9	18.0
12	11.0	2.6	3.8	4.9	
19	33.0	2.4			

showed considerable differences in iodoamino acid content compared to thyroglobulin. These data are presented in TABLES 1 and 2. In general, there was less complete iodination in S1, with a low diiodotyrosine:monoiodotyrosine ratio and a low iodothyronine:iodotyrosine ratio. This could result from a high tyrosine content of S1, a large amount of S1 available for iodination, slower

iodination of S1, or conversion of S1 to thyroglobulin prior to completion of iodination. There are no data to permit a choice among these (or other) possibilities. An iodoprotein that had physical properties similar to tumor S1 was also found in the serum of these animals. The somewhat lower D:M ratio for the hydrolytic products of serum S1 suggests that the serum S1 may be derived from tumor S1, but is secreted before completion of iodination.

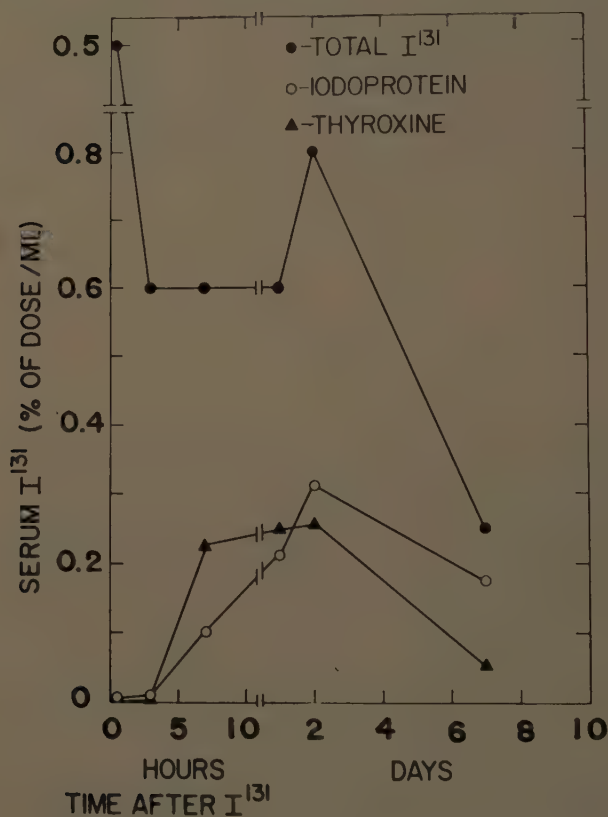


FIGURE 10. Composition of serum I^{131} of thyroidectomized rats bearing a thyroid tumor (B 310).

The amount of I^{131} in serum that is immobile in chromatography may be taken to be S1, since the presence of important amounts of thyroglobulin can be excluded. In FIGURE 10 the time course of serum radioiodine components in different rats is depicted. These rats were previously thyroidectomized so that all the serum organic I^{131} may be presumed to have originated in the thyroid tumors. It may be seen that thyroxine is released into the serum before S1, but after 1 day there is relatively more S1 in the serum than T_4 (with respect to I^{131}). These data do not appear to be compatible with the role of S1 as a precursor of the protein that releases T_4 into serum. Stable iodine analyses of serum from these rats confirmed the presence of iodoprotein, for the average

protein-bound iodine (PBI) was 10 μg . per cent, and the average BEI 2.3 μg . per cent. It is known that the PBI measures total organic I^{131} excepting iodo-tyrosines, while the butanol-extractable iodine (BEI) measures only the iodo-amino acids not linked to protein by covalent bonds.

S1 iodoproteins comparable to those in the rat tumor were also found in normal and abnormal human thyroids and in normal sheep thyroid. These differed from that in the rat, however, in electrophoresis (FIGURE 11). In man

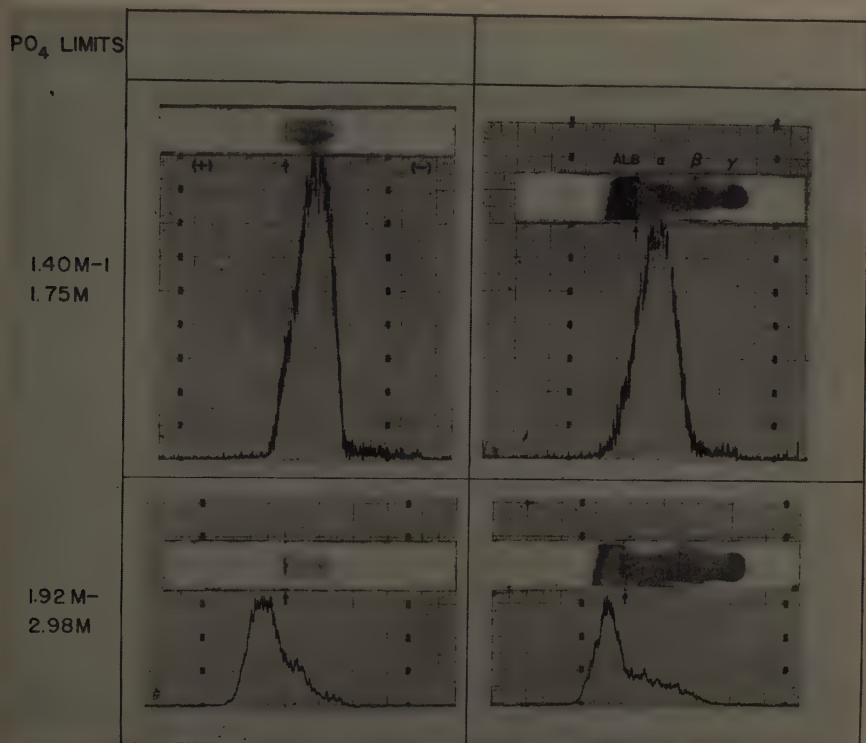


FIGURE 11. Reverse-flow electrophoresis in barbital of soluble iodoproteins from a human follicular adenoma of the thyroid. The patterns on the left are of only the isolated protein; those on the right have been added to human serum before electrophoresis.²²

its major component had a mobility like that of human serum albumin. Thus, the human thyroid S1 and blood S1 resembled each other closely. With regard to human serum S1, despite its similarity in all other respects to serum albumin, immunochemical studies¹³ demonstrated that it was antigenically different.

The quantity of S1 iodoprotein in thyroid tissue can be determined by the salting-out procedure. The data shown in TABLE 3 have been calculated from the difference in I^{131} soluble at 2.98 M from that at 1.89 M PO_4 buffer. Of the various species and types of tissue studied, only the normal rat thyroid seems to be devoid of S1. Some benign adenomas have as much as 20 per cent of the

total soluble iodoprotein in the form of S1. From the data at hand, it cannot be said whether this represents an absolute increase in the tissue content of S1 or simply a decrease in thyroglobulin.

Particulate iodoprotein. Preliminary experiments showed that from 10 to 60 per cent of the tumor I^{131} in the same strain of transplantable rat thyroid tumor (Wollman strain 1-8) was insoluble in 0.15 *M* NaCl. This insoluble I^{131} was found to increase with time after radioiodine administration and to be associated with cell particles. It was our impression at first that the radioactivity was associated with cell nuclei, since fractionation of homogenates by the Hogeboom, Schneider, and Pallade procedure in 0.88 per cent sucrose²³ showed that the major portion of the radioactivity resided in the nuclear fraction. Thyroid tumor nuclei were then prepared by a 2-phase sucrose and

TABLE 3
SOLUBLE IODOPROTEIN CONTENT OF VARIOUS TISSUES

Source	Tissue	Number of cases analyzed	Quantity of S1 percentage of organic I^{131}	
			Average	Range
Rat	Normal thyroid	4	0.1	-1.2 to +1.3
Rat	Thyroid tumor, line 1-8	12	5.2	2.7 to 9.4
Sheep	Normal thyroid	2	1.7	
Human	Normal thyroid	11	4.1	1.1 to 9.9
Human	Nontoxic nodular goiter	7	3.0	1.6 to 5.7
Human	Thyroid adenoma	4	7.8	3.5 to 19.3
Human	Chronic thyroiditis	2	5.6	4.2 to 6.9
Human	Follicular carcinoma of thyroid	4	5.0	3.2 to 6.8
Human	Solid carcinoma of thyroid	1	3.6	—
Human	Anaplastic carcinoma of thyroid	1	1.8	—

calcium method,²³ and the amount of I^{131} in the particles corresponded well with that amount insoluble in 0.15 *M* NaCl.²⁴ However, autoradiographs of thyroid tumor nuclei prepared by this method revealed that the radioactivity was confined almost entirely to extranuclear, clumped material. FIGURE 12 shows such an autoradiograph prepared from a smear of isolated nuclei. The nuclei appear to be fairly well preserved and relatively clear of silver grains, which instead are concentrated over clumped small particles. Recently D. W. E. Smith in our laboratory has attempted to discover in what type and size of particle the iodine resides. A preliminary spin of a sucrose homogenate of the tumor was done to remove soluble I^{131} . The particulate material thus obtained was layered over a denser clear layer of sucrose and sedimented at various speeds for varying times. Particles of 2 apparently discrete sizes containing I^{131} were found. FIGURE 13 demonstrates a plot of integral centrifugal force (g/min.) versus amount of I^{131} in the unsedimented phase as a derivate function showing 2 major components. There is obviously some distribution of size and/or density, however, since these 2 components usually accounted

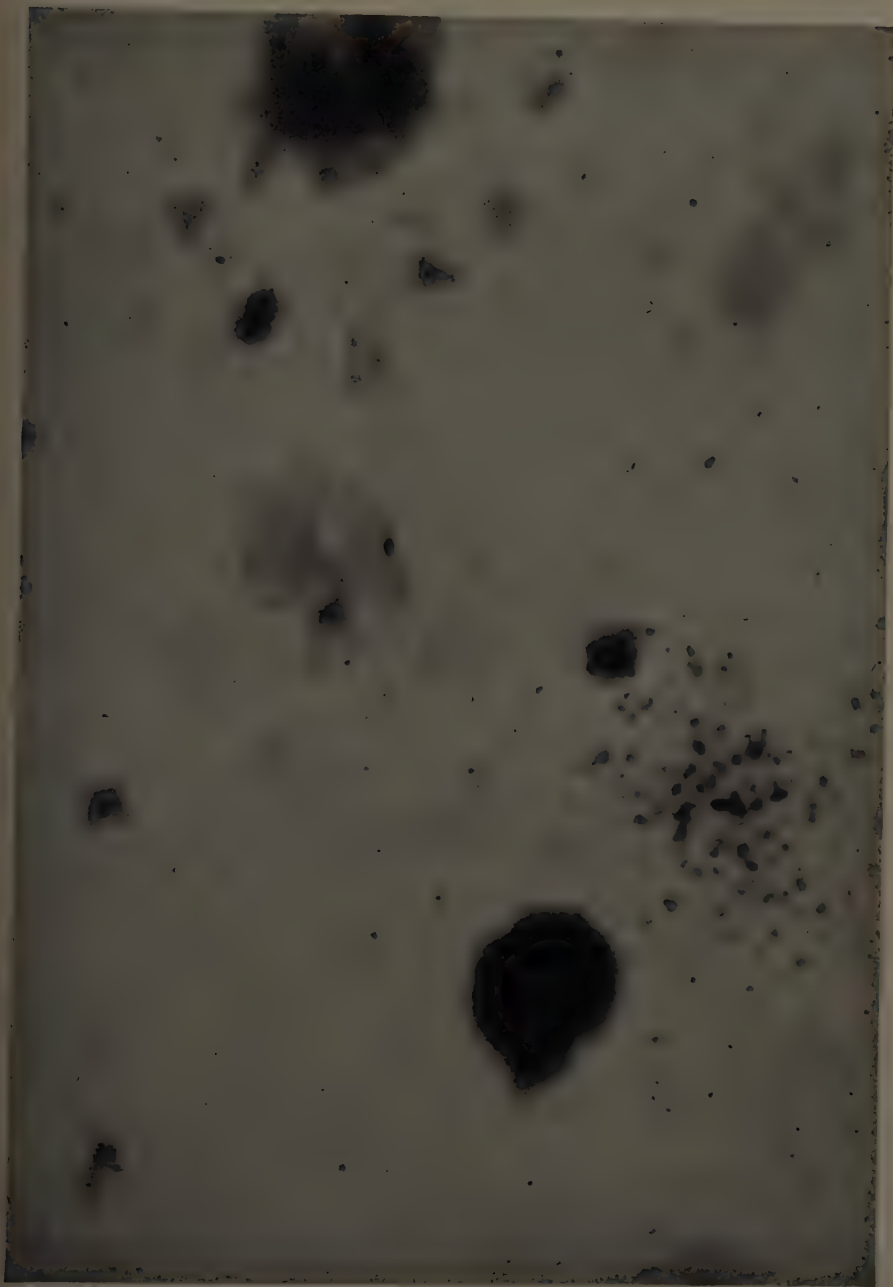


FIGURE 12. Radioautograph of a smear of "nuclei" prepared by the 2-phase sucrose method from an homogenate of rat tumor containing I^{131} .

for no more than 50 per cent of the total particulate I^{131} . Total particulate radioactivity was determined by centrifugation in 0.25 *M* sucrose at 100,000 *g* for 1 hour. L. W. Labaw of our institute has prepared electronmicrographs of these 2 fractions. It was necessary to remove the sucrose in order to obtain these, so the micrographs may not represent the particle dimensions and structure as they were isolated. The peak that sedimented more rapidly consisted of fairly uniform spheres ranging from 0.2 to 0.8 microns in diameter (FIGURE 14). The more slowly sedimenting particles appeared to be clumped to varying degrees, and an estimate of size or shape was not possible. Since they were

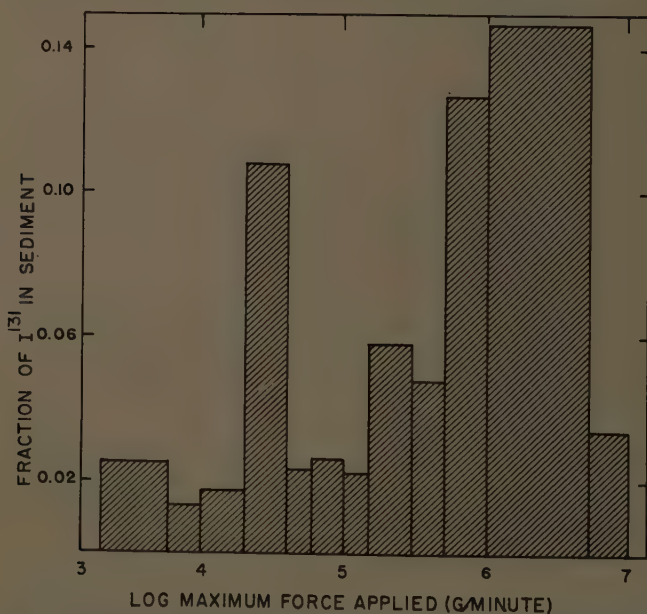


FIGURE 13. Fraction of particulate radioiodine sedimented at varying centrifugal field (g/min.). Each block represents material not sedimented by the lower force, but sedimented by the high force.

either smaller or less dense than the fast-sedimenting particles, the heterogeneity appears not to have been due to clumping per se. Other methods of sedimentation also suggested that the iodine-containing particles are not homogeneous, but it remains to be determined whether this heterogeneity is artificial.

Numerous attempts to solubilize the particulate iodoprotein were made. TABLE 4 lists the results of several types of experiments. It was found that the organic solvents used and the physical methods employed solubilized relatively little of the particulate iodine. Treatment with alkali at *pH* 13 was effective, however. FIGURE 15 shows salting-out curves for material prepared by sodium hydroxide (*pH* 13) treatment of particulate iodoprotein after preliminary extraction with 1 *M* NaCl. It is somewhat surprising that the maximum slope of radioactivity occurs at the same salt concentration as found for

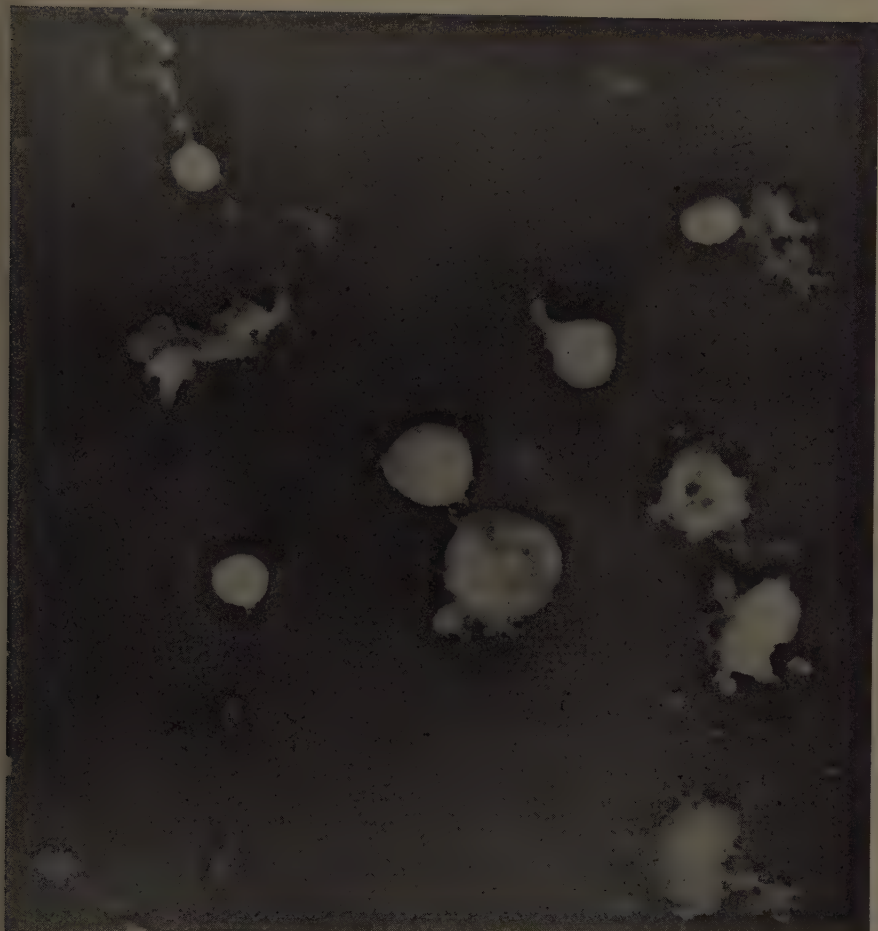


FIGURE 14. Electronmicrograph of large iodinated particle prepared in sucrose and washed. $\times 15,400$.

TABLE 4
SOLUBILIZATION OF IODOPROTEIN FROM NUCLEAR PARTICULATE (RAT TUMOR)

Method	Radioiodine solubilized Per cent of total "nuclear" I^{125}
Nossal disintegration	22
Digitonin, 0.1%	10
Desoxycholate, 0.8%	25
H ₂ O	35
NaCl, 0.15 M	<1
HCl, 0.2 M	10-15
Chloroform: methanol, 2:1	5
Ethanol, acetone, or ethyl ether	<1
NaCl, 1.0 M	15
RNase	<1
DNase	10

thyroglobulin. The range of precipitation, however, is considerably broader for alkali-solubilized particulate iodoprotein than for thyroglobulin. Electrophoresis (reverse flow) of a similar preparation with and without the addition of human serum is shown in FIGURE 16. A mobility approximately that of rat thyroglobulin is found, and there is no evidence of heterogeneity. Conventional zone electrophoresis, even with added serum, resulted in trailing on the paper. The ultracentrifugal evidence that this material is grossly different from rat thyroglobulin is presented in FIGURE 17. In these experiments the

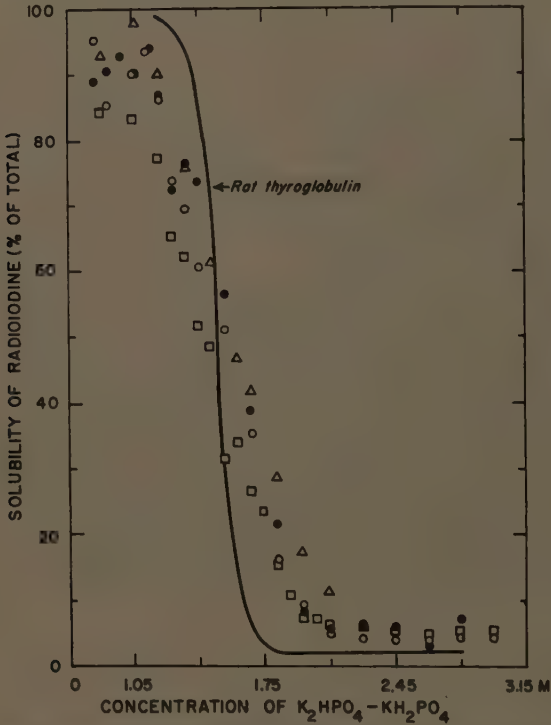


FIGURE 15. Salting-out curve of NaOH-solubilized particulate nuclear iodoprotein from a rat thyroid tumor.

solutions were sedimented in a No. 40 rotor at 39,000 rpm for 3 hours, and the contents of the tube were removed as described above. There is virtually complete sedimentation of thyroglobulin under these conditions, but the sedimentation of the solubilized particulate iodoprotein is slight and is comparable to that seen with a protein the size and shape of human serum albumin. When subjected to chromatography on diethylaminoethyl cellulose (DEAE), evidence of heterogeneity is seen. FIGURE 18 shows such a pattern when successive gradients of increasing phosphate concentration are used for elution.

More satisfactory methods for rendering the particulate iodoprotein soluble were evolved, using brief treatment with proteolytic enzymes. TABLES 5 and 6 show the effects of trypsin and pepsin. It may be seen that very brief expo-

sure to either of these enzymes apparently ruptured the critical bond or bonds involved in attachment of the iodoprotein to insoluble material. The absorption of the supernatant in the ultraviolet (280 $m\mu$) was measured after varying times of tryptic hydrolysis and indicated that the I^{131} /tyrosine ratios of the solubilized material rose for about 1 hour and then remained constant for approximately 24 hours. The soluble I^{131} was nondialyzable. FIGURE 18

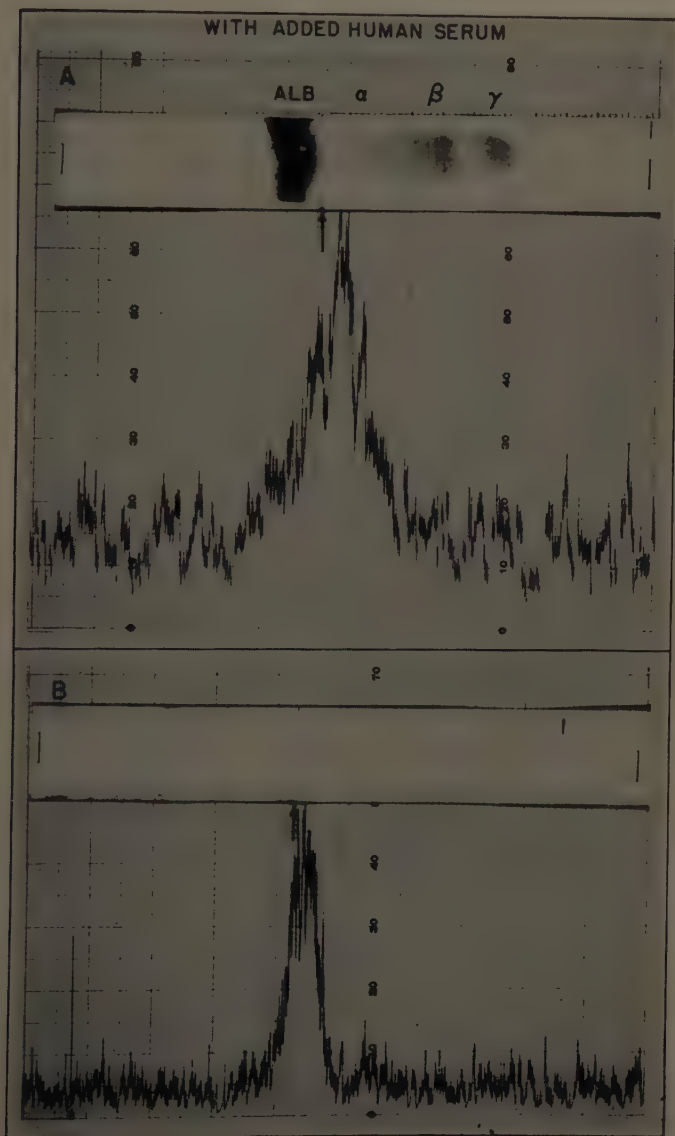


FIGURE 16. Electrophoretic mobility on paper (barbital buffer) of a NaOH-solubilized preparation of particulate nuclear iodoprotein from a rat thyroid tumor. The upper pattern is in the presence of human serum; the lower pattern is with no additions.

shows the results of chromatography on DEAE of a trypsin-solubilized preparation from a rat tumor. The rat had received I^{131} 48 hours prior to sacrifice, and the tumor was homogenized in sucrose and the particles isolated with cell nuclei by the 2-phase sucrose method. The isolated particles were extracted twice with large volumes of 1 *M* NaCl and then treated with trypsin for 30 min. The soluble phase after dialysis was chromatographed, with results somewhat similar to those obtained with alkali-solubilized material. There

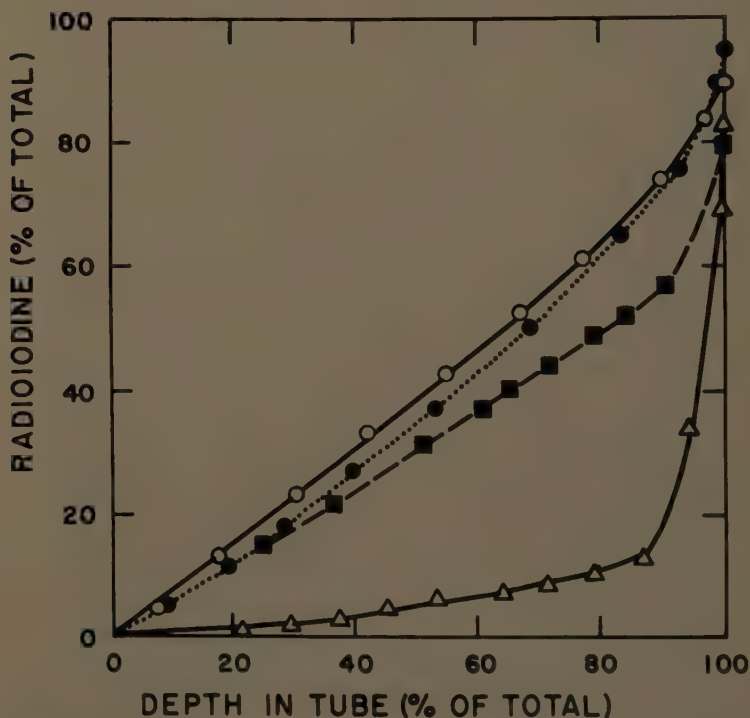


FIGURE 17. Ultracentrifuge behavior of rat thyroglobulin, NaOH-solubilized particulate nuclear iodoprotein, and trypsin-solubilized particulate nuclear iodoprotein. All materials treated in a Spinco Model L ultracentrifuge for 3 hours at 100,000 g. Symbols: open circle (○), trypsin-solubilized, pH 8, 0.1 *M* NaCl; square (■), NaOH-solubilized, pH ~7, H₂O—first spin; solid circle (●) NaOH-solubilized, pH ~7, H₂O—supernate of first spin; triangle (Δ) rat thyroglobulin.

was a major peak of radioactivity comprising over 70 per cent of the total radioiodine. This was suggestive evidence that trypsin had not split a large number of peptide bonds to give a spectrum of iodoprotein fragments.

The material in this first peak was hydrolyzed for 18 hours at 105° C. with 2*N* HCl, and quantitative amino acid analysis was done by dinitrophenylation and 2-dimensional paper chromatography according to the Levy method, using his correction factors.²⁵ Separate analyses of 3 different preparations agreed fairly well, and mean values are shown in TABLE 7. A nitrogen analysis of this preparation gave a surprisingly low figure of 3.9 per cent N. An homogenate

of rat liver was subjected to an identical procedure (isolation of nuclei, salt extraction, trypsin treatment, and chromatography), and an amino acid analysis on the resulting material is shown in TABLE 7. It may be seen that there is considerable similarity in amino acid composition between the liver and

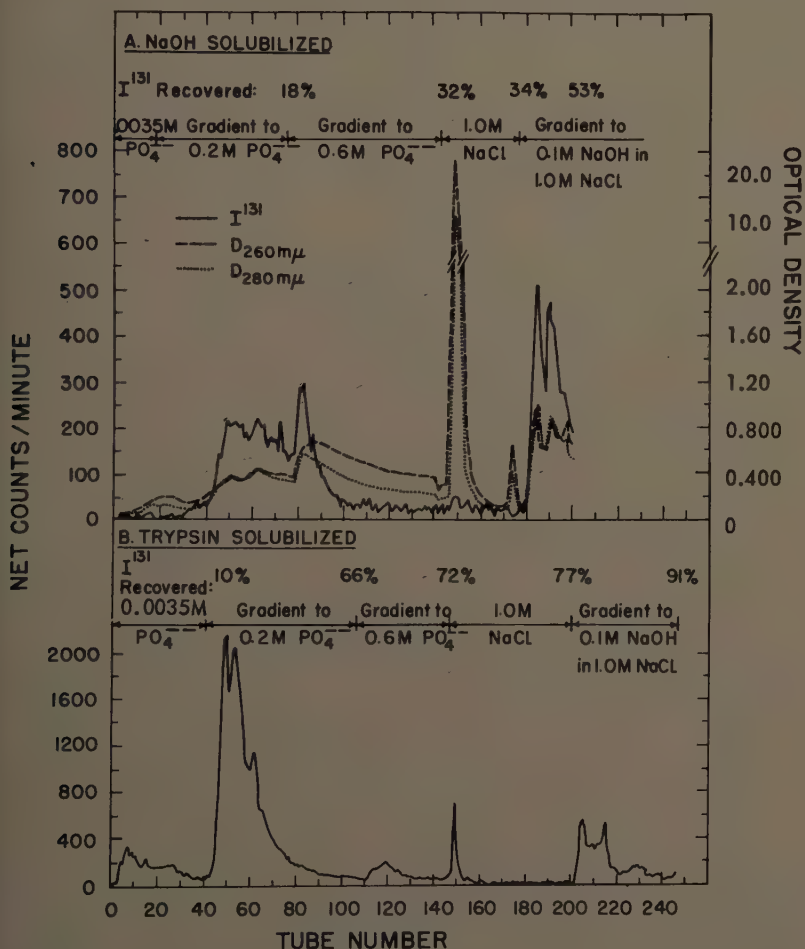


FIGURE 18. Chromatography of particulate nuclear iodoprotein from a rat thyroid tumor on DEAE cellulose.

thyroid tumor preparations. The absence of certain amino acids in the tumor, notably histidine and arginine, is worth noting. Since the solubilized tumor material had been found to have a minimal solubility at pH 4, it was not surprising to find that it had a preponderance of glutamic and aspartic acids. The very large amount of leucine present is of interest. These studies give no information about iodinated amino acids, since they are largely deiodinated by the acid hydrolysis. The extent of deiodination by this procedure was checked

TABLE 5
EFFECT OF TRYPSIN ON PARTICULATE IODOPROTEIN

Nuclei (mg.)	Trypsin (mg.)	Soluble I ¹³¹ (%)
100	0.005	38.7
100	0.010	45.4
100	0.050	53.4
100	0.100	73.0
100	0.200	74.1
100	0.400	81.0
100	0	29.8

Time: 15 min.

TABLE 6
EFFECT OF PEPSIN ON PARTICULATE IODOPROTEIN
(Nuclei from 330-mg. tissue incubated at pH 2.5)

Pepsin (mg.)	Time (min.)	Soluble I ¹³¹ (%)
10	5	86.5
10	10	89.4
10	15	91.1
10	30	91.7
0	60	22.2

TABLE 7
AMINO ACID ANALYSIS OF PARTICULATE IODOPROTEINS OF THYROID
AND LIVER AND OF THYROGLOBULINS

Acid	Rat tumor P1*	Rat liver†	Hog thyroglobulin‡
Alanine	8.6	5.7	7.4
Leucine	16.3	24.5	12.8
Valine	9.3	4.2	1.4
Glycine	13.0	11.4	3.7
Threonine	6.2	5.9	
Serine	10.9	4.0	10.8
Glutamic	12.0	14.3	
Aspartic	13.0	12.3	
Tyrosine	4.5	1.9	3.1
Diiodotyrosine			0.54
Thyroxine			0.21
Phenylalanine	3.1	7.3	6.7
Lysine	3.1	—	3.4
Unidentified		7.7	
Arginine			12.7
Histidine			2.2
Tryptophane†			2.1
Cystine			3.6
Methionine			1.3

* Listed as per cent of amino acids found.

† Not analyzed for in P1 or liver (destroyed by acid hydrolysis).

‡ From Darrien *et al.*³ and listed as per cent of protein. See Ingbar *et al.* for additional data.²⁹

with both monoiodotyrosine and diiodotyrosine, and essentially quantitative recovery as DNP tyrosine was found. Therefore, the tyrosine figure in TABLE 7 includes monoiodotyrosine and diiodotyrosine, as well as tyrosine.

Enzymatic hydrolysis (with pancreatin) was utilized for analysis of the iodoamino acids in terms of I^{131} content. FIGURE 19 shows the ratios of diiodotyrosine:monoiodotyrosine and of iodothyronines:iodotyrosines in the iodoproteins from tumors removed at different times after a labeling dose of I^{131} .

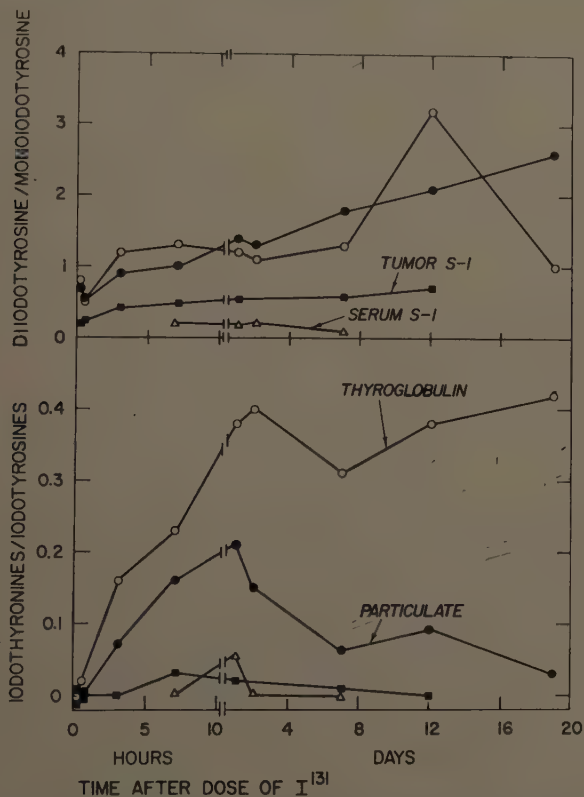


FIGURE 19. Ratios of diiodotyrosine to monoiodotyrosine and iodothyronines to iodotyrosines in several iodoproteins from a rat thyroid tumor (B 310) as a function of time after a dose of I^{131} . Reproduced by permission from *Endocrinology*.²¹

Although there was little difference in the ratios of diiodotyrosine:monoiodotyrosine in thyroglobulin and particulate iodoprotein, there were marked differences between these proteins with respect to the iodothyronine:iodotyrosine ratio, particularly at late times after the injection of I^{131} . It should be remembered that each point represents a different animal; consequently, considerable variation may be expected.

TABLE 8 shows the relative proportions of the various iodoamino acids in hydrolyzed samples of thyroglobulin and P1* in 3 rat tumors removed 2 days

* We have defined P1 as iodoprotein bound to subcellular thyroid particles and insoluble in water.

after I^{131} . The numbers represent the percentage of hydrolyzed material in each fraction except for O (origin material), which is the percentage of total I^{131} . Thyroglobulin was isolated as nondialyzable material soluble in 0.15 *M* NaCl (and hence contained a small amount of S1, see above); the particulate material was that which was insoluble in 0.15 *M* NaCl. The unknown material (U) had an R_f of 0.6 in butanol acetic acid and about 0.8 in alkaline butanol and was not identifiable as one of the known iodo amino acids. It was apparently an artifact derived from moniodotyrosine²¹ and should be added to the moniodotyrosine value in animal No. 3. TABLE 8 shows relatively good agreement among animals and, in thyroglobulin, the expected proportions of iodo-

TABLE 8
HYDROLYSIS OF THYROGLOBULIN AND PARTICULATE IODOPROTEIN*

Animal	Thyroglobulin							Particulate						
	O	I	M	D	T ₄	T ₃	U†	O	I	M	D	T ₄	T ₃	U
1	5	4	33	38	24	2	—	14	5	32	48	7	5	
2	4	8	28	29	28	4	—	14	5	37	42	7	7	
3	6	3	15	40	22	2	19	14	6	2	48	6	5	34

* See TABLE 1 for key to symbols.

† U = unidentified material.

TABLE 9
INCORPORATION OF I^{131} INTO PARTICULATE AND SOLUBLE
IODOPROTEINS OF THYROID TUMOR SLICES
Slices Transferred from Labeled to Cold Media at One Half Hour

Time (hours)	Addition	μ c. Organic I^{131}	μ c. P I^{131}	μ c. Other I^{131}
$\frac{1}{2}$	0	0.99	0.41	0.58
1	KCl	0.68	0.27	0.41
3	KCl	2.85	0.87	1.98
1	KSCN	0.89	0.33	0.56
3	KSCN	0.88	0.36	0.52

amino acids. Although the thyroxine content was low in the particulate fraction, the amount of triiodothyronine was relatively high. No explanation has been found for this phenomenon. Also to be noted is the increased amount of origin material (presumably unhydrolyzed) in the particulate fraction.

Two possibilities exist concerning the relationship between the particulate iodoprotein and thyroglobulin. Both proteins may be involved in the synthesis of thyroid hormones by distinct and unconnected routes, or they may be interrelated so that one is a precursor of the other. An attempt was made to evaluate these possibilities with an *in vitro* system (TABLE 9). Slices of rat thyroid tumor were exposed in Krebs Ringer bicarbonate medium to radioiodide for 30 min. An aliquot of the slices was removed for analysis, and the remainder was washed and divided into aliquots for removal at 1 and 3 hours. These slices were incubated in KCl, KBr, or KSCN. The first 2 were con-

sidered controls for the experiment with SCN^- in which further incorporation of I^{131} into iodoprotein was inhibited by inhibition of iodide trapping and, to a lesser extent, iodination.²⁶ It was expected that if 1 protein served as precursor of another, the relative proportions of I^{131} in the 2 materials would change. The data of Hendler²⁷ and of Peters²⁸ suggest that in oviduct and liver a particulate form of protein serves as the precursor to the final soluble protein. No such relationship was seen in our experiments (TABLE 9), and it appears that at least the iodinated amino acids in P1 and thyroglobulin are synthesized by separate pathways.

Studies have also been performed on both normal and abnormal human thyroid tissue. In all cases a variable but usually small portion of the total I^{131} was found in the particulate fraction. TABLE 10 lists the data in 21 thyroid tissues obtained from 16 different patients. In most cases the normal thyroid tissue was obtained at surgery from normal tissue adjacent to either a carcinoma of the thyroid or thyroid nodule. The average value of particulate iodine (or the "nuclear" fraction) exceeded 5 per cent of the total organic iodine except

TABLE 10
PARTICULATE IODOPROTEIN CONTENT OF SOME HUMAN THYROID

No. of cases	Tissue	Labeling	Insoluble in 0.15 M NaCl	Particulate
4	Normal thyroid	<i>In vitro</i>	6.7 *average	11.2 *average
6	Normal thyroid	<i>In vivo</i>	5.9	6.1
1	Hashimoto	<i>In vivo</i>	6.3	0.8
7	Other benign goiters	Mixed	7.1	11.3
3	Cancer	Mixed	18.0	18.2

* Percentage of total organic I^{131} .

in a single case of thyroiditis. There was no essential difference between the values obtained when the iodine in the tissue was labeled *in vivo* or *in vitro*. *In vitro* labeling was done by incubation of thyroid slices for 3 hours in Krebs Ringer bicarbonate medium containing I^{131} . As can be seen in TABLE 10, the particulate fraction was increased above normal mainly in carcinomatous tissue. The reason for this is not clear, although it is likely that, as carcinomatous tissue becomes less biochemically differentiated, it loses some enzymes responsible either for the degradation of the particulate iodine or for the synthesis of thyroglobulin. It seems less likely that a new enzyme is synthesized or that the specialized system responsible for synthesis of P1 is increased. From this standpoint the loss or diminution in level of critical enzymes in cancer may reveal intermediates ordinarily present in very small amount. Another possibility is that in tumor tissue there is loss of subcellular organization, so that proteins other than thyroglobulin may be present in a location where iodination occurs. If this were the case, however, one would expect a more random array of iodoproteins.

It was also thought of interest to determine whether the P1 fraction of human tissue was similar to the P1 of rat thyroid tumor. Normal thyroid tissue (from a patient with cancer of the thyroid) that contained 4.7 per cent of the total

I^{131} in the "nuclear" fraction was utilized. The nuclear fraction from a 2-phase sucrose-separated homogenate was extracted with 1 *M* NaCl and the sediment treated with trypsin for 30 min. About 90 per cent of the I^{131} was solubilized. This material was adsorbed on DEAE and eluted with an increasing ionic strength gradient of phosphate buffer. The results are shown in FIGURE 20, and it may be seen that the major peak of radioactivity was eluted shortly after the gradient to 0.2 *M* PO_4 was started. These results are very similar to those obtained with a trypsin-solubilized preparation from the rat thyroid tumor (see FIGURE 18). It appears, therefore, that P1 is a species of protein that can be found in a wide variety of normal and pathological thyroid tissue in several different animal species.

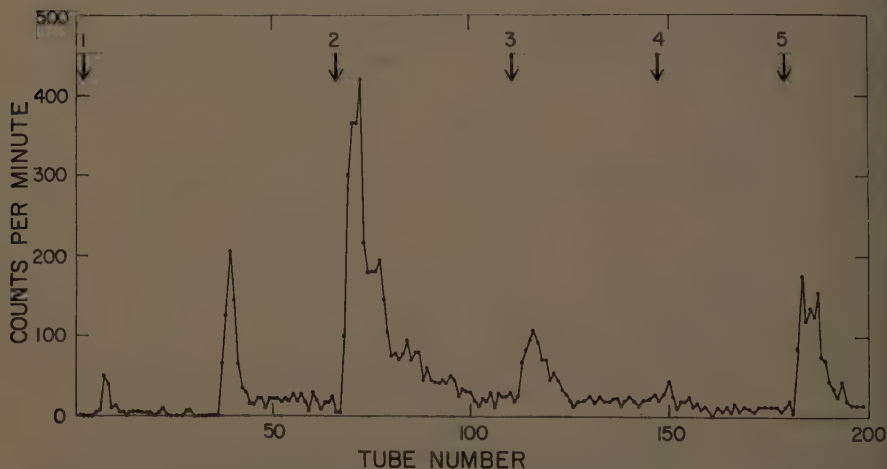


FIGURE 20. Chromatography of particulate iodoproteins from a human thyroid on DEAE cellulose. At 1 the eluting buffer was 0.0035 *M* in K_2HPO_4 - KH_2PO_4 ; at 2 an exponential gradient of the same buffer to 0.2 *M* was begun; at 3 a gradient to 0.6 *M* was initiated; at 4 the buffer was changed to 1.0 *M* NaCl; and at 5 a gradient to 0.1 *N* NaOH in 1.0 *M* NaCl was begun.

Summary

The physical characteristics of thyroglobulin have been studied after treatment with alkali and an anionic detergent. Thyroglobulin undergoes a reversible dissociation into halves, with a significant activation energy at *pH* 9.5. Above *pH* 9.5 it seems that thyroglobulin undergoes further dissociation into quarters. At *pH* 11.6 thyroglobulin becomes irreversibly denatured. Sodium dodecyl sulfate (10^{-3} *M*) causes dissociation of thyroglobulin at a neutral *pH*. In the presence of high concentrations of SDS (10^{-2} *M*) thyroglobulin and its dissociated product unfold and behave as a random coil.

Two general types of iodoproteins other than thyroglobulin have been found in normal and tumorous thyroid tissue of rats and man. One type of iodoprotein (S1) is probably of small size and is more soluble in phosphate buffer than thyroglobulin. It may be the precursor of the iodoprotein found in the blood of rats or men with certain thyroid tumors or other thyroid disease. Its rela-

tionship to normal thyroid hormone biosynthesis has yet to be elucidated. The other type (P1) is associated with subcellular particles and can be rendered soluble by strong alkali or certain proteolytic enzymes. When soluble, the protein is relatively small; it contains an excess of acidic groups and has a low content of iodothyronines. Certain physical properties of this protein have been described. It appears to be synthesized independently of thyroglobulin and is found in highest concentration in thyroid tumors.

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THE THYROID GLAND AS SOURCE AND TARGET IN AUTOSENSITIZATION*

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The proteins of the thyroid gland have been studied for several reasons. In addition to their attractiveness to endocrinologists and biochemists, they possess unusually high interest for immunologists. These antigens were first studied because they exhibited in an unambiguous fashion the phenomenon of organ specificity.^{1,2} For example, if extracts of various hog organs are tested with rabbit antiserum elicited by thyroid extract, it is found with many of these antisera that thyroid extract gives a positive result at a much greater dilution than do other organ preparations.³ More recently, the thyroid has acquired even greater interest with the discovery of its role in thyroiditis, and its capability of actually eliciting *autoantibodies*. From experimental studies of animals it was established that circulating antibodies could be demonstrated by several techniques after suitable injection of thyroid extract from within the species or even from the same animal.⁴ This condition was frequently accompanied or followed by extensive degenerative changes in the thyroid tissue—changes that, in fact, resembled human thyroiditis.⁵ Subsequent studies on a large number of human sera revealed a very high degree of correlation between the occurrence of thyroid antibodies in people with chronic nonspecific thyroiditis and its absence in other conditions or in normal serum.^{6,7}

To learn more about the character of this autosensitization response and the nature of the autoantibody, we have again immunized a group of rabbits and followed the serum changes with time. We present some of the results in this report. At the same time it has also seemed to be of importance to learn more about the antigen or antigens involved, and for this and other reasons we have been studying newer methods of purification, using hog thyroid extract as test material. Some of these results are also presented.

Production of Autosensitization

A group of 6 rabbits was immunized. For this purpose rabbit thyroid glands of 6 other rabbits were extracted as follows. The frozen glands were trimmed of fat and cut into thin slices. They were pooled and blended in a motor-driven tube homogenizer with a total of 10 ml. of saline (0.15 *M* sodium chloride). The homogenate was gently shaken overnight at 4° C. and then centrifuged for clarification. This extract was added dropwise to an equal volume of Difco Freund Complete Adjuvant fortified with a mixture of dried *Mycobacterium smegmatis* and *Myco. butyricum* in the ratio of approximately 20 mg. per 10 ml. of adjuvant. An emulsion was formed by constant mixing. After an initial trial bleeding from each rabbit, the emulsion was injected intradermally into each

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foot pad and also into 4 places in the back, using 0.1 ml. for each site. Booster injections were given similarly in the back at approximately monthly intervals, and trial bleedings were obtained at weekly intervals at first and less frequently later. The sera were stored frozen when not in use. Two of the rabbits died during the observation period; the others were exsanguinated after approximately 5 months. All thyroid glands were removed and examined histologically. The over-all schedule of injections, bleedings, and deaths is shown in TABLE 1.

All the sera were measured for total protein concentration (by the biuret method) and then examined by various means. They were studied biophysically with the aid of filter paper electrophoresis and ultracentrifugation. These results in general will not be described in any detail in the present report. The sera were also studied serologically by means of 2 procedures. The aggluti-

TABLE 1
SCHEDULE OF INJECTIONS AND BLEEDINGS

Trial bleeding	Injection	No. of weeks	Deaths
a	—	0	
—	First	0	
b	—	1	
c	—	2	
d	—	3	
e	—	4	
—	Second	4 $\frac{4}{7}$	
f	—	6	
g	—	8	
—	Third	8	
h	—	11	No. 876 at 12 $\frac{4}{7}$
i	—	14 $\frac{4}{7}$	No. 880 at 17
Final	—	20 $\frac{4}{7}$	

nation of antigen-coated tanned erythrocytes was performed in the laboratory of Noel R. Rose, by the method described in papers from our department.⁴ The alternative procedure was gel diffusion, mostly in Ouchterlony plates. These tests were performed usually with 6 wells for antisera arranged symmetrically around a central well for antigen. The gel was a 1 per cent agar, containing 0.15 *M* sodium chloride and 1:10,000 Merthiolate. A thin layer was poured into a circular steel mold of 7.5 cm. diameter resting on a glass plate. After it solidified, small steel cylinders were distributed on the surface, and agar poured around them. After the material gelled the cylinders were removed. In the earlier studies the outer wells were 1.4 cm. (edge to edge) from the central well, and each was filled with 0.20 ml. fluid. In later experiments, a smaller scale was used, with well edges separated by 0.7 cm. and volumes of about 0.08 ml. Once the wells were charged they were not filled again. The results were followed every day for at least one week, keeping the sealed plates at room temperature. Frequent sketches were drawn, and the more interesting patterns were photographed.

Results of Hemagglutination Evaluation

The first 8 bleedings (*a* to *h*) from the 6 rabbits were examined in a single experiment for their content of antibody. At a later time the final 2 bleedings (*i* and *j*) were tested, repeating the last bleeding (*h*) of the first group for comparison. These antiserum titrations were carried out with different pipettes for each of the dilution steps. The titers for the various bleedings are shown in TABLE 2. Every rabbit developed antibodies and showed a respectable titer; some titers were quite high. The gradual and steady increase with time could be noted. Furthermore, there was demonstrable antibody in 1 week after the first injection. After bleeding *h*, there was no significant increase in titer. To avoid fallacious interpretations it is imperative to compare only those results obtained within a single experiment.

TABLE 2
HEMAGGLUTINATION RESULTS: TITERS OF ALL BLEEDINGS

Trial bleeding	Rabbit 876	Rabbit 877	Rabbit 878	Rabbit 879	Rabbit 880	Rabbit 881
<i>a</i>	0	0	0	0	0	0
<i>b</i>	27	243	81	81 p	243	27
<i>c</i>	81	81 p*	243 p	243	243	81 p
<i>d</i>	243	243	810	729	729	243
<i>e</i>	243	243	2430	729	2187 p	2187
<i>f</i>	2187	2187	21,870	6561	6561	19,683
<i>g</i>	2187	6561	21,870	6561	6561	19,683
<i>h</i>	729	6561	21,870	6561	6561	19,683
<i>h</i>	7290	65,610	65,610	65,610	65,610	65,610
<i>i</i>	—	65,610	65,610	65,610	65,610	65,610
<i>j</i> (final)	—	65,610	196,830	196,830	—	196,830

* p = First bleeding to show demonstrable precipitation in gel diffusion plate.

Results of Precipitation Evaluation

The set of bleedings designated *g* was tested in a gel diffusion plate at room temperature against a saline extract of pooled, normal rabbit thyroid. On the third day, lines of precipitate appeared. A photograph of this plate is shown in FIGURE 1. There is a line for each serum except one, and they all merge perfectly. This one serum (876) was consistently nonprecipitating. It should also be noted that 881, and perhaps 880, seem to give double lines. This will be considered below. The inability to precipitate with antigen, on the part of a serum that can agglutinate antigen-coated cells, is quite often attributed to the much greater sensitivity of the agglutination technique.⁸ One would conclude that serum 876 has a titer significantly lower than the 5 precipitating sera, and it does. The hemagglutination titer of this *g* bleeding is considerably lower than the *g* from any of the other 5 series. To investigate this point further, each of the 6 sequences of bleedings was arranged for gel precipitation testing. A rather characteristic set of results is shown in FIGURE 2, which is for serum 881. Only the first bleedings, *a* and *b*, fail to precipitate. All the later bleedings show positive results with increasing density. Most of the other rabbits

in the group produced similar results, precipitation appearing in 3 days. The only exception was serum 876; it showed consistently negative results in all bleedings even after 6 days. Among the other bleeding sequences precipitation could be observed for bleedings as early as *c* in 2 others, at *e* in 1, and even as early as *b* in 1. These findings are rather interesting, for they render inadequate any attempt to explain the nonprecipitating serum on the basis of only methodological sensitivity. From an examination of the hemagglutination data (TABLE 2) again, it is clear that precipitation occurs in almost all of the sequences of sera when the hemagglutination titer is far lower than the leve



FIGURE 1. Precipitation in gel diffusion plate between rabbit thyroid extract (*central well*) and sera of bleeding *g* from the 6 rabbits (*peripheral wells*).

attained in later bleedings from rabbit 876, in which there is still no precipitation. Apparently, it is not just a question of the amount of antibody. It may be that 2 different kinds of antibody are involved. Other examples of hemagglutinating but nonprecipitating antibodies have been discussed by Davarpanah and Staub.⁹

The question of the number of lines has been studied further. FIGURE 1 indicated that 1 or 2 of the sera seemed to show double lines. Other reports have indicated a similar double-line precipitation in serum from human thyroiditis.¹⁰⁻¹³ The issue was beclouded in the present study by the fact that there was not a clear-cut separation of the lines. To check on the reality of this, one serum was set up at several distances from the antigen well. FIGURE 2 shows such a study.

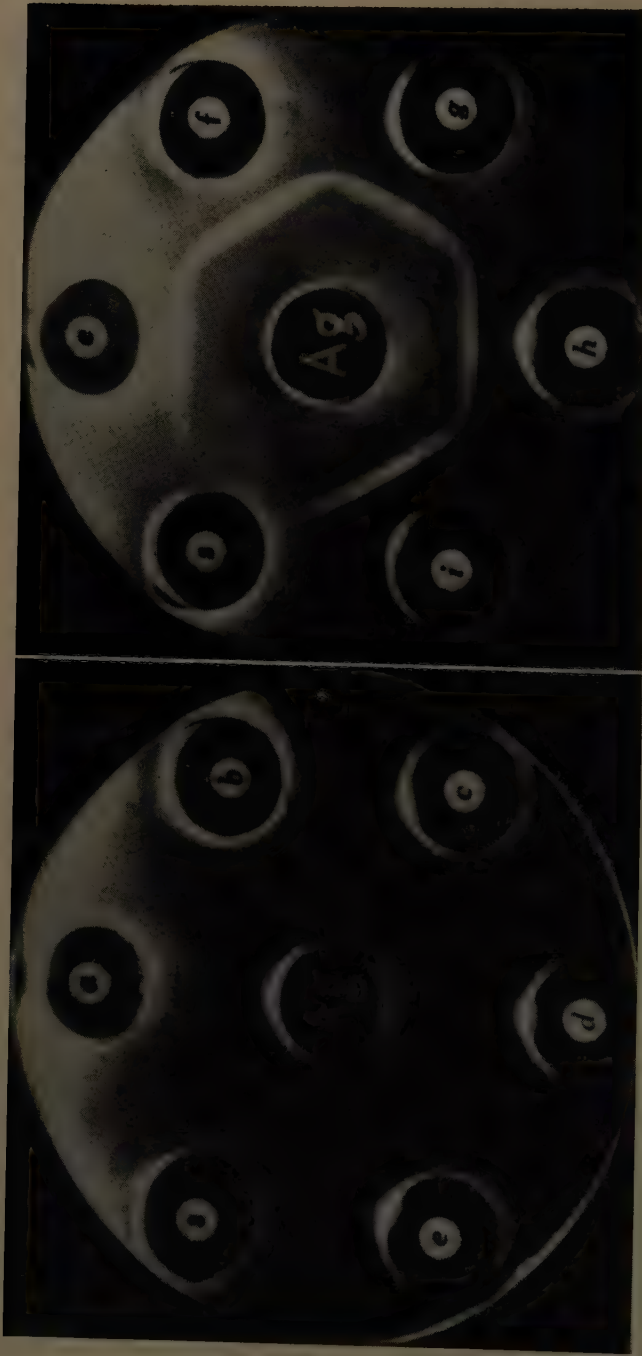


FIGURE 2. Precipitation in gel diffusion plate between rabbit thyroid extract (central wells) and sequence of sera from bleedings of rabbit 881 (peripheral wells); wells s contained saline solution.

The distances between well margins are 1.0, 2.0, and 3.0 cm. At 1 cm. a single line formed soon and remained single. At 3 cm. a very weak precipitation occurred. At 2 cm., however, a faint line appeared after some delay, and then developed into a very clear double line. On repeating this and several variations over again, it was discouraging to see the lack of reproducibility—sometimes 1 line, sometimes 2. It was finally realized that different preparations of rabbit thyroid extract had been used, and that this might be correlated with the inconsistencies of the results. The suspicion arose that the whole matter might be a reflection of the heterogeneity of the antigen prep-



FIGURE 3. Precipitation in gel diffusion plate between rabbit thyroid extract (*lower wells*) and serum 881 g (*upper wells*), with edge-to-edge separations of 1.0, 2.0, and 3.0 cm.

aration. FIGURE 4 shows a plate with a serum (878) in the center and various thyroid preparations around the periphery. In the first well is one particular crude extract and, in the next 4 wells, are fractions of this extract obtained by means of ammonium sulfate precipitation. Some can be expected to have higher proportions of thyroglobulin than the original; others, less. The relative concentrations of the major components are determined by means of analytical ultracentrifugation. The extract shows 2 lines of precipitation, as does also the first fraction—a sample with a slightly reduced content of thyroglobulin. The next 2 fractions are somewhat enriched in thyroglobulin, and they seem to show a single line. The fourth fraction, however, has a much higher proportion of components other than thyroglobulin; this again gives 2 lines. The apparent number of lines, then, is related to the proportions of the antigens.

The sixth well has crude extract from another preparation, but this also shows 1 line, although broad. This extract was made from rabbit thyroid glands kindly supplied by the Warner-Lambert Laboratories, Morris Plains, N. J., through arrangements made by Robert L. Kroc. The comparison seemed to suggest that different extracts of thyroid do give different numbers of lines. Repeated studies of this sort gave similar results. It has been observed by some¹⁴ that double lines can result from particular concentration relationships. Accordingly, additional plates were made with both antigen and antiserum



FIGURE 4. Precipitation in gel diffusion plate between rabbit serum 878, final bleeding (*central well*), and various rabbit thyroid preparations (*peripheral wells*). Well 1, crude extract of laboratory rabbits; well 2, fraction precipitated between 0.00 *M* and 1.50 *M* ammonium sulfate; well 3, fraction precipitated between 1.50 *M* and 1.60 *M*; well 4, fraction precipitated between 1.60 *M* and 1.70 *M*; well 5, fraction precipitated between 1.70 *M* and 4.00 *M*; well 6, crude extract of Warner-Lambert rabbits.

diluted by various ratios. Other plates were incubated with more stringent thermal control to check on the possibility of a temperature anomaly. Despite all these attempts, the observation seemed to remain valid, namely, samples with lower thyroglobulin percentage than an initial extract consistently showed 2 separated lines. Since it has been strongly indicated that thyroglobulin is the (main) antigen corresponding to the human autoantibody,¹⁵ it seems reasonable to implicate it similarly in the rabbit system. The present study suggests that a second antigen can also elicit autoantibody formation, or that there is a cross reaction with it. This second antigen might be 1 of the minor components, although not necessarily one of the 4 detected in the ultracentrifuge.

Clearly, however, further study will be necessary before a good understanding of this double precipitation and of the antigens involved is reached.

Results of Histological Study

We have discussed the thyroid gland as a source of antigens leading to auto-sensitization. A more detailed discussion of these antigens will appear below. The role of the gland as a target will now be considered. The gland from each of these rabbits was examined post-mortem. The tissues were fixed and then stained with hematoxylin and eosin. The thyroid glands of all 6 rabbits showed

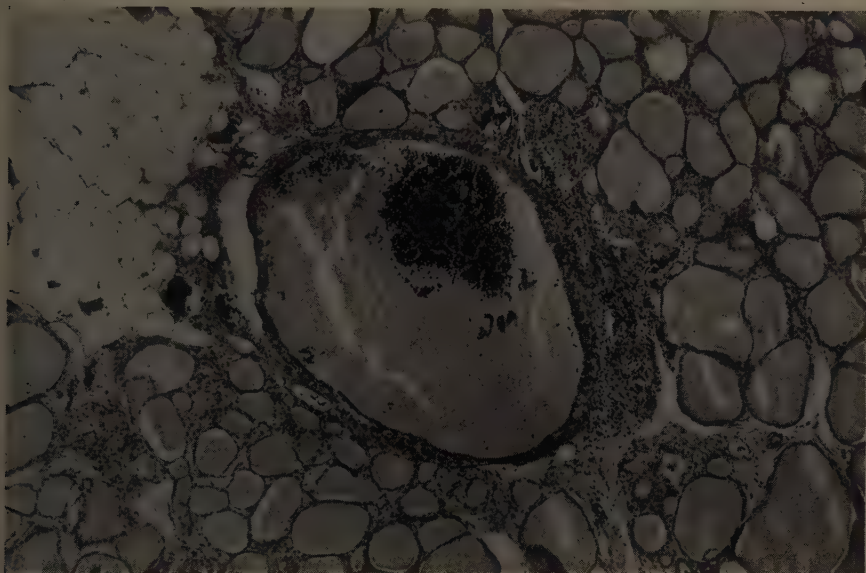


FIGURE 5. Histological specimen of thyroid tissue from rabbit 876. Only very slight abnormality. The somewhat eccentrically located cystic duct contains some desquamated epithelium and many lymphocytes. Infiltration by a few plasma cells and lymphocytes in the surrounding stroma.

damage. The range of alterations can be illustrated with 3 examples. FIGURE 5 is taken from rabbit 876, and reveals only very slight abnormality. FIGURE 6, from rabbit 877, shows very distinct changes with extensive infiltration of lymphocytes and eosinophils. FIGURE 7, from rabbit 881, discloses very marked changes, including widespread fibrosis. We are indebted to K. Terplan, University of Buffalo School of Medicine, Buffalo, N. Y., for these interpretations. It is highly tempting to try to correlate the degree of histological damage with the antibody titer as revealed by hemagglutination. The observations for these 3 rabbits would fit very well, but the data are inadequate. A much more extensive survey along these lines has been published describing work done in our laboratories.¹⁶

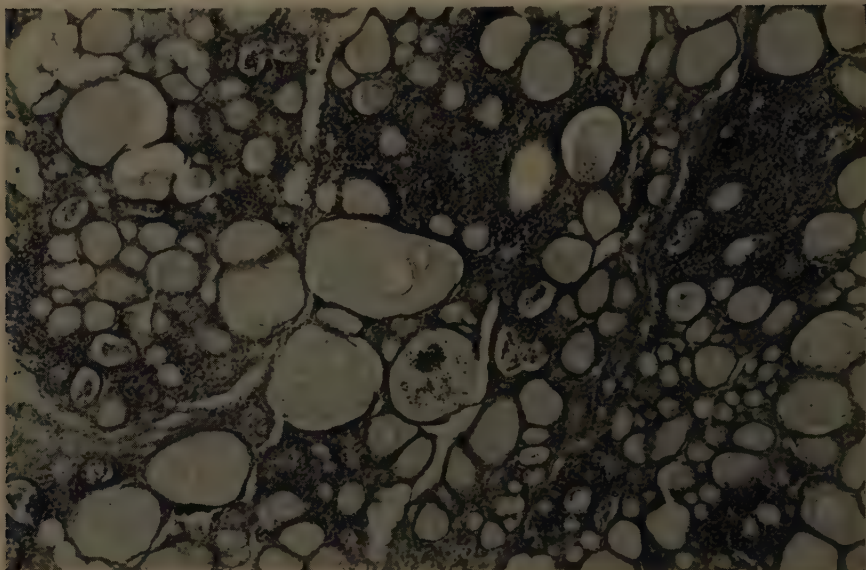


FIGURE 6. Histological specimen of thyroid tissue from rabbit 877. Very distinct changes, with dense focal infiltrations by lymphocytic and eosinophilic cells and by many disintegrating leukocytes. Numerous macrophages and desquamated epithelial cells intermingled with leukocytes in the lumen of the follicles; there is also nuclear debris.

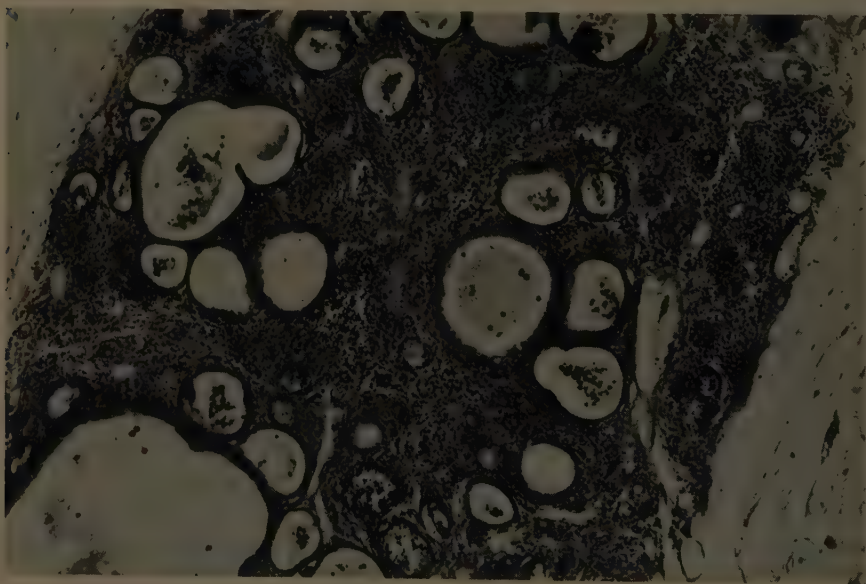


FIGURE 7. Histological specimen of thyroid tissue from rabbit 881. Very marked changes with extensive fibrosis and cystic distention of follicles, which contain much nuclear debris and many macrophages, some with hemosiderin granules. Many small atrophic follicles without any colloid.

Nature of Thyroid Extract

Attempts to purify the extracted proteins of thyroid tissue have been made with a number of procedures. Here we shall describe primarily our recent efforts with column chromatography.

First, let us better understand our starting material. Since hog thyroid glands are larger and more easily obtained in quantity than are rabbit glands

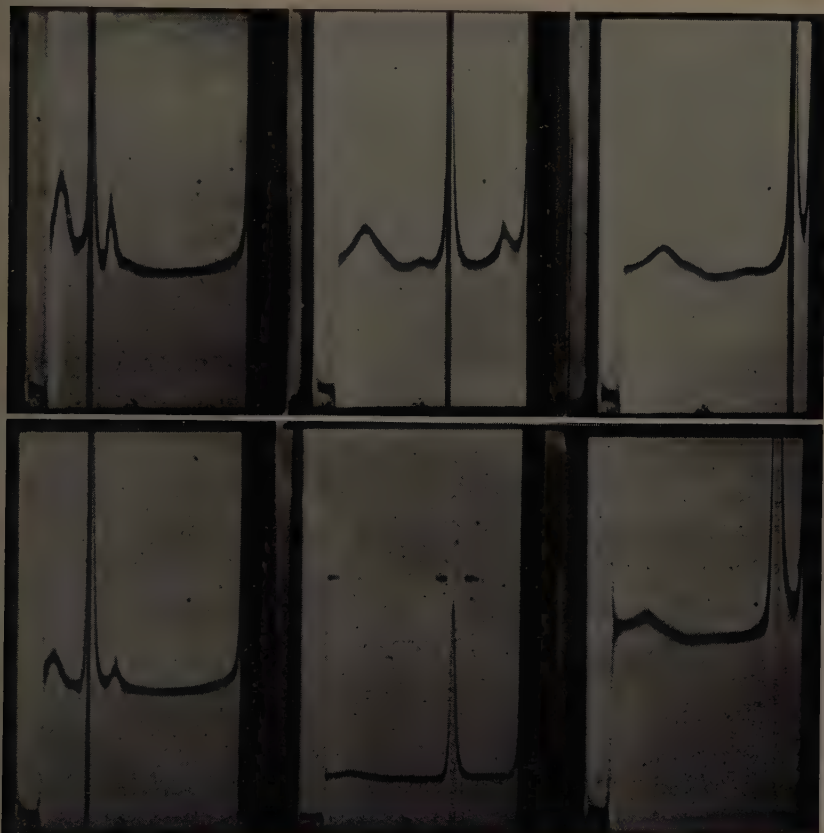


FIGURE 8. Ultracentrifugal patterns of hog thyroid crude extract (preparation 96) *Upper line:* protein concentration, 2.00 per cent; photographs at 8, 26, and 44 min. after rotor reached speed of 58,200 rpm. *Lower line:* protein concentration, 0.80 per cent; photographs at 8, 26, and 34 min. after rotor reached speed of 59,700 rpm.

most of the work on methods has been done with such material. A saline extract of hog thyroids, when examined in the analytic ultracentrifuge (Spinco Model E), shows 5 sedimenting components. Typical patterns are shown in FIGURE 8.

Some time ago these 5 peaks were named I, II, III, IV, and V, in decreasing order of sedimentation rate.^{17,18} Peak II has an extrapolated sedimentation rate of 18.7 *S*, and is identified as thyroglobulin. The others are of quite unknown nature. Similar pictures are seen for other species. Rall and his co-

workers have also shown or described such patterns, confirming the presence of all 5 of these constituents and obtaining virtually the same sedimentation constants for them.¹⁹⁻²¹ The proportion of the thyroglobulin peak in crude hog thyroid extracts is about 80 per cent of the sedimenting material. This is an important figure as the base line for evaluating the success of fractionation. If one uses optical electrophoresis (Spinco Model H) at pH 8.6 in 0.10 ionic strength barbital buffer, a pattern similar to that in FIGURE 9 is seen. There are 4 boundaries designated *A*, *B*, *C*, and *D*, again at decreasing speeds of migration. The dominating one, *B*, corresponds to approximately 92 per cent of the total. It has a mobility in the range of serum α -globulin, as previously



FIGURE 9. Electrophoretic pattern (descending) of hog thyroid crude extract (preparation 96). Protein concentration, 0.65 per cent; barbital buffer, pH 8.60; ionic strength, 0.10; electric field strength, 6.77 volts/cm.; time, 180 min.

determined from filter paper electrophoresis of hog extract¹⁷ and optical electrophoresis of human extract.¹⁸

Purification of Thyroid Extract

Earlier studies in this laboratory have been made on fractionation by ammonium sulfate precipitation.^{22,23} While details have not been published, the essence of the procedure is to collect the fraction precipitating in the range of 1.60 to 1.70 *M* ammonium sulfate and then, after reconcentrating the supernatant and removing salt by dialysis, to reprecipitate in the range of 2.50 to 3.00 *M* ammonium sulfate. The first fraction is about 95 per cent of peak II, or thyroglobulin. The second fraction is 100 per cent of peak V. Because of its solubility properties, this latter material was named thyralbumin.²³ It was

found, however, to be quite heterogeneous on electrophoretic examination; it was found also to contain iodine, as stated by Robbins *et al.*²⁴

In our more recent attempts to purify such extracts, we have employed columns of diethylaminoethyl cellulose in a procedure similar to that of Sober *et al.* for serum proteins.²⁵ The studies have been carried out by Peter Stanley in our laboratory. The saline extract of hog thyroid glands is dialyzed against the initial buffer and then applied to the column. A gradient elution procedure is used whereby the salt concentration is increased and the *pH* is decreased by introducing a series of sodium phosphate-sodium chloride buffers with salt concentrations ranging from 0.005 *M* to 0.15 *M* or higher and *pH* values ranging from 7.2 to 4.2. The buffer descriptions are given in TABLE 3. The *pH* values of the developing buffer are deceptive, however, and do not correspond to the *pH* levels actually found in the effluent. This effluent was collected at a flow rate of up to 1 ml./min. in fractions of 6.1 ml. with a volumetric fraction collector. The concentration of protein in each fraction is indicated by the optical density reading at 280 μ . In the first-column fractionation of a thyroid extract, an elution diagram was obtained, as shown in FIGURE 10. Here we had

TABLE 3
BUFFERS FOR COLUMN CHROMATOGRAPHY OF THYROID PROTEINS

1. 0.005 <i>M</i> Na phosphate	<i>pH</i> 7.2
2.* 0.02 <i>M</i> Na phosphate	6.0
3. 0.05 <i>M</i> NaH ₂ PO ₄	4.5
4.* 0.05 <i>M</i> NaH ₂ PO ₄ + 0.02 <i>M</i> NaCl	—
5. 0.05 <i>M</i> NaH ₂ PO ₄ + 0.05 <i>M</i> NaCl	4.5
6. 0.05 <i>M</i> NaH ₂ PO ₄ + 0.10 <i>M</i> NaCl	4.4
7. 0.05 <i>M</i> NaH ₂ PO ₄	4.2

* Omitted from the series in later studies.

put 1.6 gm. of protein on 29 gm. of adsorbent. A fairly sharp and tall peak comes off at the very start, and then there is a lengthy succession of buffer changes with no release of protein. Finally, a second very broad peak is delivered. Nothing more seemed to follow. At first it was thought that the early material could be divided into 2 parts and the latter 1 spread into 3 parts, but later it was felt that there was insufficient justification for this, and there are now considered to be only these 2 major regions of protein, designated *A* and *D*. One sometimes sees 2 very small peaks in the intermediate region. Chromatographic studies of thyroid proteins have also been made by Ingbar *et al.*²⁶ The results showed some differences from ours, as well as some similarities. For one thing, Ingbar and his colleagues apparently obtained only a single early peak of protein material in the effluent. However, they had started with a partially purified thyroglobulin rather than with crude extract. The best material that they isolated seemed to be fairly pure in thyroglobulin, although the concentration at which the ultracentrifugal evaluation was made was not stated.

In later runs we put more protein (4.0 gm.) onto the column, and we sharpened the second peak by making the salt gradient steeper, as can be seen in FIGURE 11. A portion of the pooled peak *A* was then put on a fresh column and run through again. This is also reported in FIGURE 11.

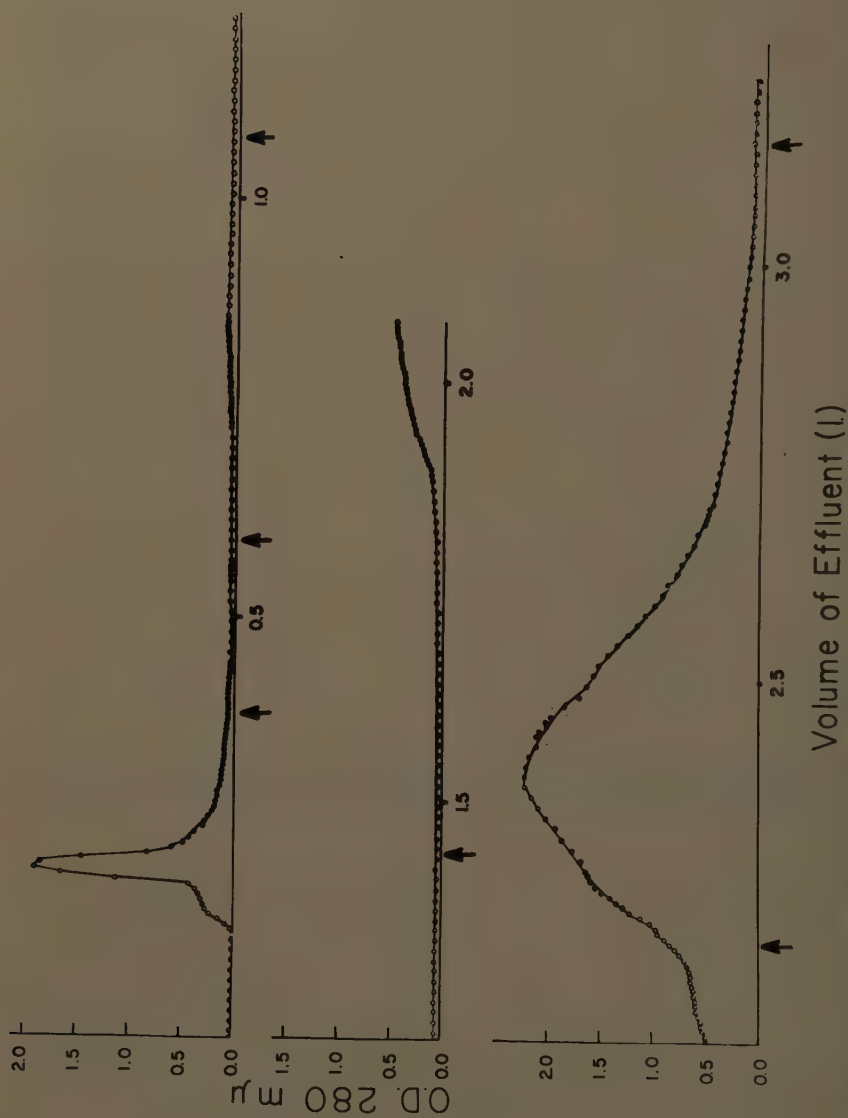


FIGURE 10. Optical density plotted against effluent volume for chromatography of hog thyroid crude extract (run 3).

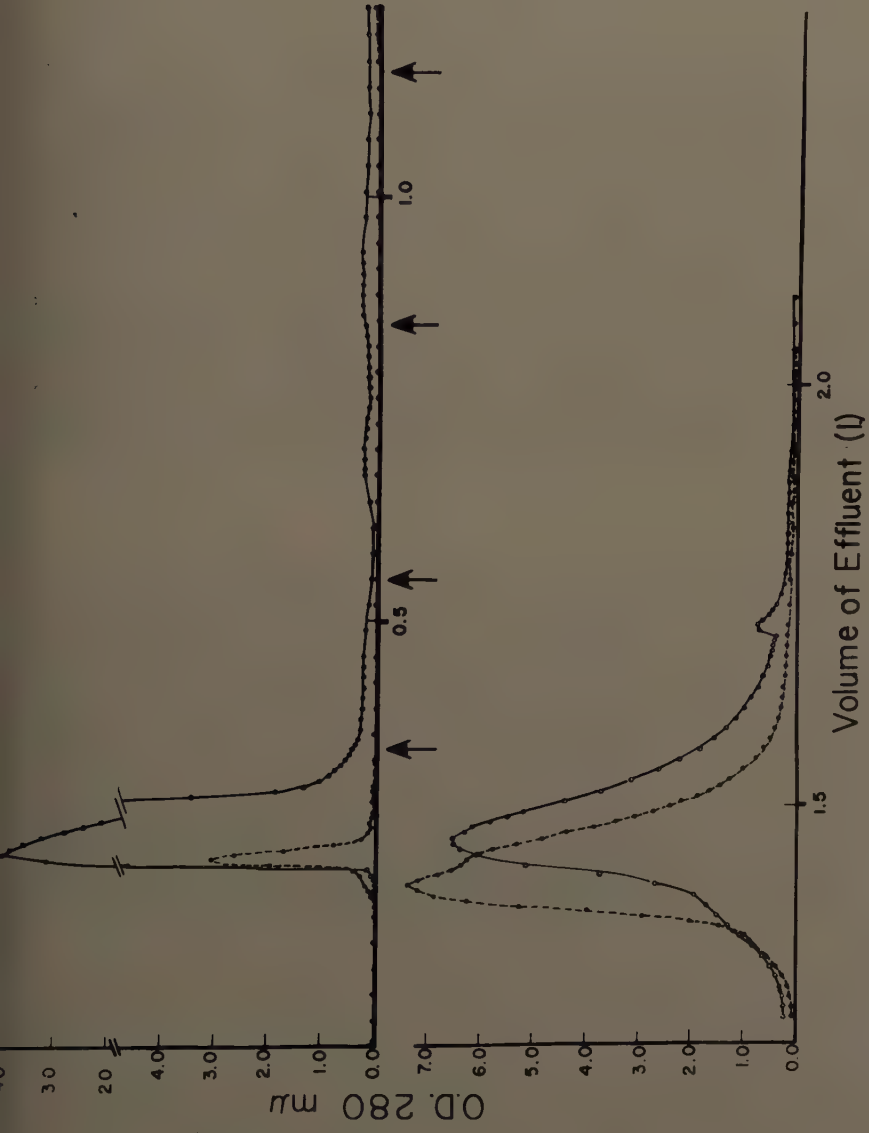


FIGURE 11. Optical density plotted, against effluent volume for chromatography of hog thyroid crude extract (run 8, open circles), and of peak A from run 8 (run 9, filled circles).

Many ultracentrifugal studies have been made on these eluted samples. In general, both peak *A* and peak *D* were predominantly thyroglobulin, as was the original extract. For example, in column run 3 (FIGURE 10) peak *A* gave material with 84 per cent of 19 *S* component and peak *D* gave 75 per cent of 19 *S*, while it was 78 per cent in the original extract. This is rather puzzling, as is

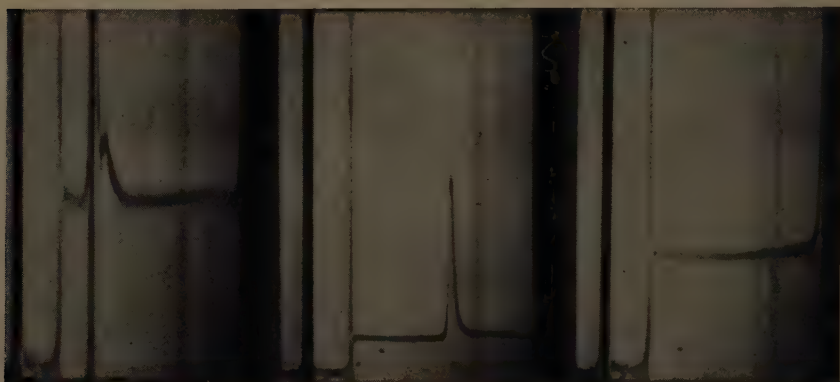


FIGURE 12. Ultracentrifugal patterns of peak *A* material from column run 8. Protein concentration, 0.70 per cent; photographs at 4, 18, and 32 min. after rotor reached speed of 59,800 rpm.

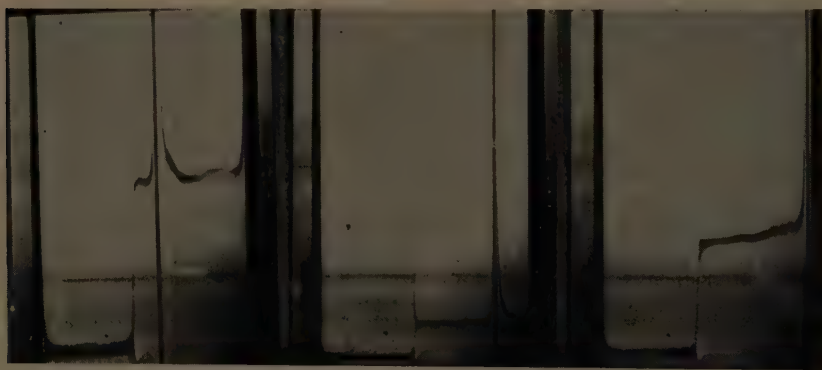


FIGURE 13. Ultracentrifugal patterns of separation cell top component obtained from peak *A* material of column run 8. Protein concentration, 0.81 per cent; photographs at 4, 16, and 22 min. after rotor reached speed of 59,500 rpm. The base line imperfection (near cell bottom) should be ignored.

also the lack of any discovered effluent in which other constituents predominate. It is also strange that a large fraction of the peak *A* material shifts to peak *D* position on rerunning. One can easily postulate a nonspecific "breakthrough" of unbound protein, but the total amount employed in relation to the size of column is even smaller than was used in serum studies.²⁵ However, in a recent peak *A* sample, column 8 in FIGURE 11, the product was a material with 89 per cent thyroglobulin and 11 per cent fast component, but no sign of slow peaks, as is shown in FIGURE 12. This looked like such a good removal of the slow-

sedimenting components that we then decided to remove the fast one by use of the separation cell of the ultracentrifuge. It was therefore centrifuged at 2.2 per cent, the undiluted concentration, in the fixed partition cell, and the upper solution was withdrawn after a suitable time. This gave an exceedingly small yield, and it became necessary to pool the supernatants of several successive runs to provide 2 or 3 ml., but the product as shown in FIGURE 13 was quite gratifying. This was run at 0.8 per cent and seems to be close to being thyroglobulin of 100 per cent ultracentrifugal homogeneity.

We have more recently been studying a stepwise elution scheme, with somewhat different buffer characteristics. This procedure shows promise of producing well-purified thyroglobulin, and also some of the other constituents. It has been tried with rabbit thyroid extract and has given similar results.

We should therefore soon be in a position to repeat our studies on rabbit and human autoantibodies to the thyroid gland with the additional advantage of using really purified thyroglobulin as an isolated antigen.

Acknowledgments

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DEIODINATION OF THE IODINATED AMINO ACIDS*

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When iodotyrosines or iodothyronines labeled with I^{131} are administered intravenously to normal man, most of the labeled iodine appears in the course of time in the urine as free inorganic iodide. Only negligible amounts of the undegraded parent substances pass the renal barrier intact. Evidently, there are mechanisms in the body that can remove iodine from the aromatic rings of these physiologically important substances and in the process reduce it to its ionic form. Much effort has attended the study of these processes of deiodination, for it has become apparent that they may be critical in ordering the metabolic impact of these compounds. Indeed, deficiencies of deiodination may underlie profound disturbances of growth and development.^{1,2}

The problem of deiodination has been approached in many laboratories from many different points of view. Seemingly discordant results may be attributed to differences in technique and, perhaps more significantly, to differences in the events being measured. This review will survey the pathways and mechanisms of deiodination of the aromatic amino acids and their chemical relatives, as indicated by information obtained from both *in vivo* and *in vitro* studies.

Aromatic Iodine Bond and Deiodination

Iodine in the 3 position of the phenolic ring of the iodinated amino acids probably differs little in its intrinsic stability from the iodine of iodophenol, which is quite stable. The bond energy of approximately³ 45 kcal./M is less than that of hydrogen and, accordingly, the structure is less stable than the parent compound. The bond is formed by a shared pair of electrons from the outer shells of carbon and iodine.

A major factor in the chemical reactivity of any of these compounds is the easy ionizability of the hydroxyl group. The ether linkage of iodothyronines is not ionized and, accordingly, there is more stability of the 3 and 5 positions of the alpha ring than there is of the 3' and 5' positions. Accordingly, one might expect the 3 and 5 positions to be more stable than the 3' and 5' positions. This expectation is borne out by studies of deiodination in the rat.^{4,5} Steric factors may contribute significantly to any deiodination sequence.

Iodination and deiodination of phenol in its simplest terms is probably analogous to the reversible iodination of ketones. Iodine formed from iodide by oxidation

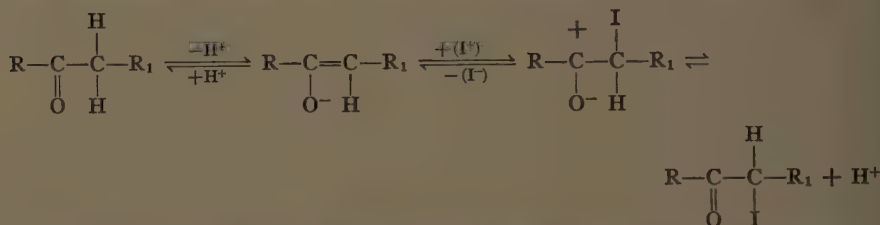


* The investigation reported in this paper was supported in part by research grants from the Public Health Service, Bethesda, Md., and by the American Cancer Society, New York, N.Y.

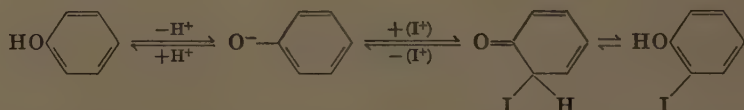
undergoes in the course of iodination heterolytic fission to form an iodide ion and the hypothetical iodonium ion



where each bar represents a pair of unshared electrons. Aliphatic ketones may enolize and take up an I^+ , leaving a proton and iodide ion; thus

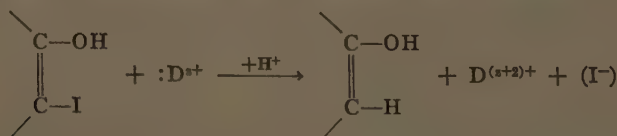


Similarly, the reversible iodination of iodophenol proceeds through an intermediate anion:



Phenol loses a proton to form an anion (second compound), which in turn acquires an iodonium ion to form the third compound. This then enolizes to the iodophenol. Conditions that facilitate the removal of I^+ (or I_2) drive the reaction to the left (deiodinization) and, contrariwise, those that provide I^+ (or I_2) drive it to the right.

The reaction of deiodinization may be summarized schematically as follows:



where $:\text{D}$ is any nucleophilic ion such as $:\text{I}^-$, $:\text{S}^-$, $\text{S}_2\text{O}_3^{2-}$, or an electron donor such as Fe^{++} , Cu^+ , or a hydride ion.

This reaction scheme is relevant only to the relatively straightforward displacement of iodine. Some of the deiodination reactions may involve more complex reactions of hydroxylation leading to ring rupture and would be more difficult to diagram. These may be related in principle to the aromatic hydroxylations of many substances described by Udenfriend *et al.*⁶ and requiring ferrous ion, ascorbate, and oxygen. I shall return to this subject in detail later.

Thyroxine (T_4) and triiodothyronine (T_3) are rapidly deiodinated upon exposure to high-energy gamma radiation.⁷ The products of the deiodination are iodide and two iodinated compounds, one of which is the lactic analogue. This finding of Tata has been confirmed in our laboratory in a 5000-c. cobalt unit. The decay of samples of T_4 of high specific activity from internal radia-

tion has been troublesome, but largely can be obviated by diluting and refrigerating the labeled T_4 upon receipt of the relatively pure product. Degradation of iodotyrosines in the dry state by alpha particles has been observed by Yalow.⁸

Recently an important reversible "deiodination" of labeled T_4 has been observed by Tata.^{9,10} Sudden dilution of I^{131} -labeled T_4 , T_3 , 3-5-diiodotyrosine (DIT), 3-monoiodotyrosine (MIT), and 3,5- T_2 from aqueous organic solvents caused an apparent rapid loss of part of the radioactivity from organic binding, followed by a gradual reappearance of the labeled compound. The phenomenon was observed at alkaline pH, and re-emergence of the parent compound was greatly accelerated by acid. Although the product as tested chromatographically was indistinguishable from iodide, other observations indicated that the product was not iodide. Presumably there was an ionization of the phenolic hydroxyl and reversible molecular rearrangement to a compound with chromatographic character similar to that of iodide in the alkaline solvent system employed. The change is inhibited by many tissue proteins, best by those that best bind the labeled compound. These observations are of particular importance to the present discussion because of the possibility that some of the dehalogenations described may have been in fact phenomena related to the physicochemical changes of the kind described by Tata.

Deiodination of Mono- and Diiodotyrosine

The first careful studies demonstrating *in vivo* deiodination of DIT were those of Oswald¹¹ in 1910 who, after oral administration to animals, found that much of the iodine appeared in the urine as iodide and an unidentified organic compound of iodine. Foster and Gutman¹² fed rabbits daily 1 to 2 gm. of DIT. Sixty per cent was recovered unchanged in the urine, 10 per cent as iodide and 18 per cent as the lactic acid derivative of DIT. Deiodination in liver brei was demonstrated by Hartmann.¹³ Detailed studies of a thyroidal enzyme that employs both MIT and DIT as substrate were described in a series of papers by Roche *et al.*^{14,15} The enzyme was found in a variety of tissues, including thyroid, liver, and kidney. MIT was an intermediate.

Tong *et al.*¹⁶ found that I^{131} -labeled DIT was metabolized readily by rat liver and kidney slices and that the principal products were iodide and the pyruvic and lactic analogues of DIT. An unidentified product was also found. Homogenates failed to metabolize DIT unless fortified with pyruvate or α -ketoglutarate, and the principal products were the unknown compound and the pyruvic analogue. Thyroid slices deiodinated readily, but iodide was the principal product even when the system was fortified with pyruvate or α -ketoglutarate. Deiodination of DIT also was observed after intravenous administration to rats, but small amounts of the lactic and pyruvic analogues were demonstrated in the blood. Similarly, Hartmann^{17,18} has observed deiodination of DIT in homogenates of swine liver. He found that the deiodination took place in a soluble fraction of the homogenate, and that a fraction sedimenting between 25 and 40 per cent $(NH_4)_2SO_4$ contained the active principle. The yield was small and the incubation was for many hours.

Studies in this laboratory with tissue iodotyrosine deiodinase were stimu-

lated by the finding of several patients with familial goiter and hypothyroidism who were unable to deiodinate MIT and DIT. Not only did these substances and certain derivatives appear in the peripheral blood, but significant deiodination of administered labeled MIT and DIT failed to occur.¹ Earlier, Albert and Keating¹⁹ had shown that, after intravenous administration of DIT to man, most of the iodine appears in the urine as iodide. This process was confirmed and was found to include MIT.²⁰ The significant products appearing in the urine of these goitrous subjects after administration of I¹³¹ were MIT, DIT, and at least one other labeled compound. After intravenous administration of labeled MIT to these subjects, two zones of labeled iodine in addition to MIT appeared in the chromatograms of urine. One of these was easily hydrolyzed to MIT. The other, in another patient from the laboratory of Querido, has been identified recently by Michel as the acetic acid derivative of MIT.* Subsequent studies²¹ with thyroid slices from the gland of one of these subjects failed to indicate any deiodinating capacity for DIT.

Studies with tissue homogenates of thyroid, liver, and kidney have disclosed that both MIT and DIT are deiodinated by a thermolabile enzyme of the microsomes that specifically requires reduced triphosphopyridine nucleotide (TPNH) as its co-factor.²²⁻²⁴ A TPNH-generating system employing the glucose-6-phosphate dehydrogenase of the microsomes is equally effective. The enzyme is thermolabile at 55° C. and is inhibited by parahydroxymercuribenzoate and not by cyanide. It does not require oxygen. Other properties of what is presumably the same enzyme have been described by Slingerland and Josephs.²⁵ The enzyme is easily demonstrated in liver and kidney homogenates only when nicotinamide is added to prevent undue destruction of the nucleotide, but this supplement is not required in thyroid preparations. Iodination of C¹⁴-labeled tyrosine has provided MIT and DIT, both C¹⁴-labeled, which upon deiodination in the system yield C¹⁴-labeled tyrosine.^{23,24} Thus, the ring is not ruptured, nor is the side chain deaminated in this deiodination. Presumably this enzyme is the principal, if not the only, physiological deiodinating enzyme for the iodotyrosines.

Deiodination of labeled DIT is inhibited when stable MIT is added, and vice versa. These kinetic studies were consistent with substrate competition, and suggest that both substrates are deiodinated by the same enzyme. An approximate Michaelis constant k_m for DIT is 9.2×10^{-7} and for MIT is 3.7×10^{-7} . Thus, the iodine of MIT is removed more easily than the first iodine of DIT.

Hartmann¹⁸ suggested that deamination of DIT is a necessary preliminary step in its deiodination. Tong *et al.*¹⁶ and Nakana *et al.*,²⁶ using the amine oxidase of snake venom, have demonstrated deamination of iodotyrosine as well as deiodination, but the finding of Tong *et al.* that pyruvate-fortified liver homogenate deaminates without deiodinating indicates that deiodination is not an inevitable consequence of oxidative deamination. The relatively small amount of iodinated derivatives of MIT and the virtual failure of any derivatives of DIT to appear after their administration to patients who are

* I am indebted to Querido of the University of Leiden, The Netherlands, and to Michel of the University of Paris, France, for permission to make reference to this finding.

unable to deiodinate these substances suggest that oxidative deamination is a minor metabolic pathway of both.

In summary, it may be said that the iodotyrosines are deiodinated rapidly by the intact animal. This is accomplished by a deiodinase, or by deiodinases, of wide distribution. One of these is a heat-labile microsomal enzyme that requires TPNH as a co-factor. Alternative and probably minor pathways

TABLE 1
FATE OF IODOTYROSINES *IN VIVO*

Species	Substrate	Product	
		Blood	Urine
Normal man	MIT DIT	I ⁻	I ⁻
Certain cretins	MIT DIT	MIT, conjugates and derivatives DIT	MIT, conjugates and derivatives DIT
Rabbit	DIT		I ⁻ , lactate
Rat	DIT	Pyruvate, lactate	I ⁻
Rat (eviscerated)	DIT		I ⁻

TABLE 2
FATE OF IODOTYROSINES *IN VITRO*

Tissue	Substrate	Product	Conditions
Liver slices Kidney slices	DIT	I ⁻ , pyruvate and lactate, MIT, tyrosine	
Liver homogenate	MIT, DIT DIT	I ⁻ Pyruvate	+ Nicotinamide + Pyruvate
Thyroid microsomes	MIT, DIT	I ⁻ , MIT, tyrosine	+ TPNH
Thyroid slices	DIT, MIT	I ⁻ , MIT, tyrosine	
Snake venom	DIT, MIT	I ⁻ , pyruvate, acetate	

of metabolism that are visible under certain circumstances both *in vivo* and *in vitro* include formation of the lactic, pyruvic, and acetic analogues, and these in turn may serve as substrates for deiodination or be deiodinated through their innate instability. The metabolism of the iodotyrosines under various circumstances is outlined in TABLES 1, 2, and 3.

Deiodination of Iodothyroacids in Vivo

Thyroxine. After administration of labeled T₄ to normal man, the half time of disappearance from the blood is 6 to 7 days.^{27,28} The principal meta-

bolic product that may be identified in the blood or urine is inorganic iodide (TABLE 4). There is controversial evidence^{29,30} regarding the appearance of T_3 in man after administration of T_4 . Roche *et al.*³¹ failed to identify T_3 in the urine or bile of rats given T_4 intraperitoneally, but demonstrated the α -keto derivatives of T_4 in urine and of T_3 in bile when the parent substances were given. They considered oxidative deamination the first step in liver degradation of these substances. In the hepatectomized dog no T_3 derivatives were formed from T_4 , and T_3 was found only rarely after T_4 .³² Traces of a sulfur conjugate of T_3 have been found in the bile after administration of T_4 to rats.³³ Gross and Leblond³⁴ found that T_4 is converted to T_3 in the thy-

TABLE 3
FATE OF IODOTYROSINES FROM THE POINT OF VIEW OF THE
COMPOUNDS METABOLIZED

	Product
MIT	
<i>In vivo</i>	
Normal man	I ⁻
Certain cretins	MIT, conjugates and derivatives
<i>In vitro</i>	
Thyroid slices	I ⁻ , tyrosine
Liver homogenate + nicotinamide	I ⁻
Snake venom	I ⁻ , pyruvate, acetate
DIT	
<i>In vivo</i>	
Normal man	I
Certain cretins	DIT
Rabbit	I ⁻ , lactate
Rat	I ⁻
Rat, eviscerated	I ⁻ (slowly)
<i>In vitro</i>	
Rat liver and kidney slices	I ⁻ , pyruvate, lactate, tyrosine
Rat liver homogenate	I
Rat liver homogenate + pyruvate	Pyruvate
Rat liver and kidney microsomes + TPNH	I ⁻ , tyrosine
Thyroid slices	I ⁻ , tyrosine
Snake venom	I ⁻ , pyruvate, acetate

roidectomized rat and that iodide appeared in its urine. Kalant *et al.*^{35,36} reported the appearance of T_3 in muscle and liver after T_4 was injected into normal and propylthiouracil-treated rats, and Ford *et al.*³⁷ have identified T_3 consistently in many tissues of the guinea pig after administration of T_4 . Hogness *et al.*³⁸ detected T_3 in approximately 20 per cent of analyses of liver, kidney, and muscle after intravenous administration of labeled T_4 . Indirect evidence of *in vivo* deiodination of T_4 to T_3 has come from one laboratory.³⁹⁻⁴¹ The appearance of iodide in the urine of rats after both T_4 and T_3 is retarded by administration of butyl-4-hydroxy-3-5-diiodobenzoate, but this substance inhibits the response of the metabolic rate to administration of T_4 and accentuates the response to T_3 .

Peripheral pathways of metabolism other than deiodination are open to T_4 and are discussed in detail elsewhere in this publication.

Klitgaard *et al.*⁴² prepared DL-T₄-1-C¹⁴ and found after its injection into the rat that 20 per cent was in the bile after 14 hours, 10 per cent in the expired air, and only 1 per cent in the urine. Thus, decarboxylation may be a major pathway of T₄ metabolism, but it is not yet clear whether this proceeds *in vivo* independently of deiodinization. A glucuronide of T₄ has been demonstrated abundantly in the bile under a variety of circumstances, but glucuronides of T₃ do not appear⁴³⁻⁴⁷ after administration of T₄. Galton and Pitt-Rivers⁴⁸ have found a conjugate of T₄ in the kidneys of mice given I¹³¹ and have also identified the acetic analogues of T₃ and T₄ in liver and kidney.⁴⁹ The conjugate was probably a glucuronoconjugate rather than a sulfate ester.

TABLE 4
FATE OF IODOTHYROACIDS *IN VIVO**

Species	Substrate	Product				
		Blood	Urine	Liver	Muscle	Kidney
Normal man	T ₄	?T ₃	I ⁻			
	T ₃		I ⁻			
	3,3'-T ₂		I ⁻			
	3,3',5'-T ₃		I ⁻			
Rat	T ₄	T ₃	I ⁻ , pyruvate	T ₃	T ₃ TRIAC, I ⁻ , 3,3'-T ₂	TRIAC, I ⁻ , 3,3'-T ₂
	T ₃		I ⁻ , glucuronide			
	TETRAC		I ⁻			
	TRIAC		I ⁻			
	3,3'-T ₂		I ⁻			
	3,3',5'-T ₃					3,3',5-T ₃ 3,3'-T ₂ 3,3'-T ₂ - acetate
Guinea pig	T ₄			T ₃		T ₃

* Glucuronoconjugates and keto acid analogues of the respective iodocompounds appear in the bile, usually along with several unidentified iodinated substances, and in the rat the sulfur conjugate of T₃ has been reported after administration of T₄.

Triiodothyronine. The half life of administered labeled T₃ in normal man is 2.5 days,⁵⁰ and iodide is the principal form in which the labeling iodine appears in the urine. Flock *et al.*⁵¹ found iodide in the urine after administration of T₃ to dogs with biliary fistulas but, with biliary obstruction, a glucuronoconjugate and two unidentified compounds appeared in the urine. Traces of DIT and MIT were said to appear in the bile of these animals. Roche *et al.*³¹ found the keto acid derivative of T₃ in the bile of rats after intraperitoneal administration of T₃. Iodide and 3:5:3'-triiodothyroacetic acid (TRIAC) and 3,3'-T₂ appeared in the kidney^{31,52-54} and muscle.³⁸ It appears, as suggested by Roche *et al.*,⁵⁴ that T₃ may have 2 principal pathways, of widely different metabolic implications, one to the metabolically active and, perhaps, key substance, TRIAC, and the other to the useless degradative 3,3'-T₂ intermediate. Conceivably, control of the balance between

these 2 competing pathways is critical in determining the peripheral effects of the hormone. Of several components of rat bile⁵⁵ after administration of T_3 , the principal one appeared to be identical to a component also observed in plasma by Gross *et al.*⁵⁶ and thought to be a complex of T_3 : probably a glucuronide or sulfate ester produced in the liver for transport to the muscles. It was detected first in the liver and kidney, and then in the plasma, but the muscle component was in the uncomplexed form as free T_3 . A sulfur conjugate of T_3 appears in small amounts in the bile of the rat⁵⁷ after administration of T_3 .

Other iodothyroacids. TRIAC is largely confined to the vascular compartment after parenteral administration. Much of the iodine appears as iodide in the urine.^{58,59} Roche *et al.*⁵³ found this component widely distributed, but in highest concentration in the kidney and largest amount in the liver. The concentration per gram of organ was high compared to that in the blood. It appears in the bile as a glucuronoconjugate.⁶⁰

The other iodothyronines, $3,3'$ - T_2 and $3,3',5'$ - T_3 , which have been identified in the thyroid and serum of the rat,⁶¹⁻⁶³ are rapidly degraded to iodide when administered intravenously to normal man.^{64,65} The acetic acid derivative of $3,3'$ - T_2 is found in the kidney after administration to thyroidectomized rats.⁶⁶ It is concentrated temporarily in the liver, and then is eliminated rapidly. Much appears as iodide in both urine and bile. The glucuronide also has been identified.⁶⁷ Minute amounts of $3,3'$ - T_2 and its acetic analogue, and of $3,3',5'$ -TRIAC are found after administration of $3,3',5'$ -triiodothyronine,⁶⁶ and large amounts of a glucuronoconjugate appear in the bile.⁶⁸

The isolated perfused rabbit liver degrades T_4 to several components, including iodide.⁶⁹ The most abundant was thought to be a glucuronide, but T_3 was identified in some but not all experiments. Several unidentified components and iodide appeared also in the perfusate and bile after perfusion with a solution containing labeled T_3 .^{69,70} The perfused rabbit kidney metabolizes T_4 to T_3 .⁷¹ No T_3 appeared in the perfusate, but it was identified in extracts of the organ after completion of the perfusion.

Thus it appears, as pointed out by Roche *et al.*,⁵⁴ that in the intact animal there are three pathways of metabolism open to the iodothyronines, which may undergo simple stepwise deiodination, glucuronyl conjugation, or oxidative deamination and decarboxylation. These pathways are not mutually exclusive. Factors that determine the predominating pathway and the precise mechanism of each degradative step remain to be ascertained.

Metabolism of Iodothyroacids by Tissue Slices

Albright and his colleagues in 1954 first described the metabolism of T_4 by kidney slices of the rat.⁷² Slices incubated for 3 or more hours in Krebs-Ringer phosphate at pH 7.4 were found to produce an iodinated component that, when chromatographed in the butanol-ammonia solvent system, corresponded to T_3 (TABLE 5). Slices from hyperthyroid animals metabolized the T_4 more rapidly than those from normal ones, and slices from hypothyroid animals scarcely at all.⁷³ An adaptive T_4 deiodinating enzyme of kidney was postulated. The system was inhibited by 3×10^{-7} M thiouracil and by adding T_3 to the medium, and was heat-labile. These findings have been

challenged on the grounds that, in the chromatographic system employed, T_3 and tetraiodothyroacetic acid (TETRAC) are distinguished with difficulty. The latter compound has been demonstrated in the kidney after administration of T_4 to the intact animal.⁷⁴ This objection has been answered by Larson *et al.*,⁷⁵ who employed a tertiary amyl alcohol-ammonia solvent system that is said to resolve the two compounds. It is noteworthy that Albright *et al.* have used extremely low concentrations of T_4 in demonstrating the deiodination to T_3 . Cruchaud *et al.*⁷⁶ have also demonstrated the formation of T_3 in kidney slices incubated with T_4 . Etling and Barker⁷⁷ failed to observe the appearance of T_3 in kidney slices incubated for 3 days with T_4 , although a large fraction of T_4 was deiodinated to iodide.

The principal product of the incubation of liver slices with T_4 is iodide.⁷⁸ Yamazaki and Slingerland⁷⁹ found that rat liver slices incubated for 3 hours deiodinated approximately 50 per cent of T_4 . Diiodination was inhibited

TABLE 5
FATE OF IODOTHYROACIDS IN TISSUE SLICES AND PERFUSED ORGANS

Tissue	Substrate	Product
Human white cells	T_4	I^-
Liver slice	T_4	I^- , TETRAC, thyronine
	T_3	I^- (slowly)
	TETRAC, TRIAC	I^- (slowly)
	TETRAPROP TRIPROP	I^-
Kidney slice	T_4	I^- , T_3
Perfused rabbit liver	T_4	I^- , T_4 -glucuronide, T_3 , etc.
	T_3	I^- , etc.
Perfused rabbit kidney	T_4	T_3 (in kidney)

by anaerobiosis, Hg^{++} , *n*-butyl-4-hydroxy 3:5-diiodobenzoate, and to a lesser degree by ethylenediaminetetraacetic acid tetrasodium salt (EDTA) and cyanide. TETRAC was formed by liver slices. T_3 did not appear. Lissitzky *et al.*⁷⁸ incubated liver slices with T_4 made chemically and by iodination *in vivo*. The *in vivo*-labeled substrate was much less actively deiodinated (10 per cent) than that labeled in the 3':5' position (63 per cent). Small amounts of TETRAC were identified. No T_3 , 3,3'- T_2 , or iodotyrosines could be identified. The product of deiodination by liver slices has been identified by an ingenious indirect method as thyronine.⁸⁰

Liver slices deiodinate T_3 less rapidly than T_4 . Yamazaki and Slingerland⁷⁹ found that only 0 to 10 per cent of TRIAC and TETRAC was deiodinated. TRIPROP and TETRAPROP, the respective propionic analogues of TRIAC, were deiodinated somewhat more. Intermediate products of these deiodinations have not been detected or identified in liver slice preparations. Slices of organs other than liver and kidney have not been studied extensively. Deiodination of T_4 by human and rabbit white blood cells has been observed recently by Kurland and Hamolsky.⁸¹

In summary, liver and kidney slices deiodinate T_4 , T_3 , and their propionic acid analogues. The acetic analogues are deiodinated more slowly. It is not agreed entirely that T_3 is an identifiable deiodination product of T_4 in kidney or liver slices. It has been observed only by Albright *et al.*,⁷² and by Cruchaud *et al.*,⁷⁶ employing the same techniques. The acetic acid analogue of T_4 appears after incubating T_4 with liver slices. Thyronine is said to be a product of the deiodination of T_4 .

TABLE 6
FATE OF IODOTHYROACIDS IN TISSUE EXTRACTS AND HOMOGENATES

Tissue	Substrate	Product
Kidney homogenate	T_4	I^- , TETRAC
Solubilized mitochondria	T_4 T_3	TETRAC TRIAC
Liver homogenate	T_4 , T_3 TETRAC, TRIAC TRIPROP TETRAPROP	I^- I^- (slowly) I^-
Solubilized mitochondria	T_3	TRIAC
Liver "extract"	T_4 T_3 TETRAC, TRIAC	I^- , ? T_3 , DIT conjugate I^- I^- (slowly)
Brain homogenate	T_4 T_3	I^- , TETRAC* I^- , TRIAC*
Brain homogenate (Hg^{++})	T_4 T_3	TETRAC* TRIAC*
Rat muscle	T_4 , T_3 TETRAC, TRIAC TETRAPROP TRIPROP	I^- I^- (slowly) I^-
Snake venom	T_4 , T_3	Acetic analogues

* Or propionic acid analogue.

Deiodination of Iodothyroacids and Related Compounds by Tissue Homogenates and Extracts

Homogenization of kidney tissue eliminates the deiodination of T_4 ; instead, the principal product is TETRAC (TABLE 6). Similarly, TRIAC is formed if the substrate is T_3 , but iodide is also formed.^{75,82-84} No T_3 appears when T_4 is substrate, but at least two substances other than TETRAC (or TRIAC) appear, and these are not deiodination products. The active enzyme for decarboxylation was solubilized by sonic treatment of a kidney mitochondrial preparation. Microsomes and soluble fractions were not effective. Homogenates heated to 100° C. for 10 minutes were inactive. D- T_4 was not metabolized.⁷⁶ When the homogenate was dialyzed, its activity was doubled by the addition of diphosphopyridine nucleotide (DPN).

Deiodination of T_4 by liver homogenates has been observed by many investigators.^{79, 85-89} Yamazaki and Slingerland⁷⁹ found that deiodination of endogenously labeled T_4 was complete; accordingly, they concluded that no significant amount of DIT or compound labeled at the 3 position is formed as an intermediate, except transiently. This result is in accord with that of Lissitzky *et al.*,⁸⁰ who found that thyronine is the end product of hepatic deiodination. On the other hand, Plaskett,⁸⁹ using T_4 labeled at the 3 position, found that the product of deiodination by dilute homogenates of liver is a compound easily hydrolyzed to DIT. On present evidence, there appears no way to resolve this seeming conflict.

T_3 is also deiodinated by homogenates of liver,⁸⁸ as are TRIAC and TETRAC, but the last two much less readily than T_4 or T_3 in the homogenate system.^{79, 88} The propionic acid analogues were deiodinated almost as readily as the parent iodothyronines.

The metabolism of T_3 by rat mitochondria has been examined in detail by Albright *et al.*⁹⁰ Deiodination was most active in preparations from liver and kidney, but was also observed in heart, spleen, and brain. When the mitochondria were solubilized in the sonic oscillator, deiodination was sharply reduced, and TRIAC formation was enhanced.

T_4 metabolism by homogenates of brain tissue has been studied extensively by Tata.⁹¹⁻⁹³ There was no noteworthy difference between slices and whole homogenate. In whole brain homogenate, T_4 was deiodinated somewhat more rapidly than T_3 . The enzyme was most abundant in the cerebral cortex. Two metabolic events were observed in these preparations, a deiodination and a deamination. The deiodinase for both T_4 and T_3 was principally in the supernatant fraction of the homogenate, whereas deamination was confined principally to the mitochondria. Little deiodination was found in the particulate fractions. In controls there was no deiodination after 30 min. of boiling. The deiodinase was unaffected by anaerobic conditions and was not inhibited by CN^- . Hg^{++} inhibited the deiodinase, but not the deaminating enzyme. The principal product formed was iodide, but under appropriate conditions of deiodinase inhibition TETRAC and TRIAC (or TETRAPROP and TRIPROP, which would not have been differentiated chromatographically) were formed, respectively, from T_4 and T_3 . Because no T_3 was formed from T_4 , Tata *et al.*⁹¹ tentatively formulated a preferred degradative scheme through the deaminated analogues prior to deiodination in brain tissue. However, it may be that T_3 is by-passed in T_4 deiodination. Furthermore, it will be recalled that TETRAC and TRIAC are degraded more slowly by liver than are the corresponding amino acids. It would be of interest to know the relative rates of degradation of these substances when directly measured in brain homogenate.

Homogenates of muscle have yielded a deiodinase similar to that in brain,^{92, 93} but there is evidently no deaminase in muscle. Deiodinase activity is confined to the soluble fraction of the muscle homogenate. Muscle deiodinates faster than brain, and T_4 is degraded faster than T_3 . The deiodinase activity of both muscle and brain differs from the liver system of MacLagan⁸⁶⁻⁸⁸ in that it proceeds anaerobically and is labile to boiling at 100° C. for 10 min. The major product of muscle deiodination proved to be iodide. TRIAC was not identified as a product of T_3 incubation, nor was 3:3'- T_2 .

A report of a muscle deiodinase for thyroxine activated by ferrous ion and potentiated by flavine nucleotides has just appeared from the laboratory of Tata.⁹⁴

To summarize, the acetic acid analogues of T_4 and T_3 are formed upon incubation of their parent iodothyronines in kidney homogenates. The enzyme has been solubilized from the mitochondria. A soluble deiodinase and a deaminase for T_4 and T_3 can be obtained from brain. A deaminase for T_4 and T_3 is not present in muscle, but a deiodinase for T_4 , T_3 , and their propionic and acetic analogues has been found. A dilute strained homogenate of liver and several other tissues deiodinates T_4 and T_3 . One of the products has been identified as an iodinated compound easily hydrolyzed to DIT. There has been a noteworthy failure to identify T_3 as a product of T_4 deiodination in

TABLE 7
THE METABOLIC FATE OF THYROXINE

	Blood	Urine	Liver	Kidney	Muscle
T_4					
<i>In vivo</i>					
Normal man		I ⁻			
Rat	T_3	I ⁻ , pyruvate	T_3	T_3	TETRAC
Guinea pig			T_3		T_3
<i>In vivo</i>					
Liver slice			I ⁻ , TETRAC, thyronine		
Kidney slice			I ⁻ , T_3 (?)		
Perfused rabbit liver			T_3 , T_4 glucuronidase, I ⁻ , etc.		
Kidney homogenate			I ⁻ , TETRAC		
Kidney mitochondria			TETRAC		
Liver homogenate			I ⁻		
Liver "extract"			I ⁻ , DIT conjugate(s), ? T_3		
Brain homogenate			I ⁻ , TETRAC		
Muscle			I ⁻		
Snake venom			TETRAC		

homogenate preparations. These metabolic events are summarized in TABLES 7 to 9.

The Heat-Stable Liver Deiodinating System

The foregoing discussion has summarized the high spots of our knowledge of the deiodinating systems of metabolizing mammalian tissues. Discussion of the heat-stable liver deiodinating system first described by Maclagan⁸⁶⁻⁸⁸ has been carefully skirted. The system is an interesting if unusual one that will now be considered in more detail.

Maclagan initially described a deiodination observed in whole homogenates of liver tissue that had two *pH* optima far to the acid and alkaline sides and that was resistant to boiling for many min. Activity gradually declined until, after 2 hours of boiling, it had nearly disappeared.⁷⁷ The *pH* effect appeared to be an artifact of the poor solubility of T_4 near the neutral point for, when tracer amounts of labeled T_4 were used, the *pH* optimum was between

5 and 7. Subsequently, a somewhat different preparation was described. It was made by mincing liver tissue and filtering it through gauze, and was less heat-resistant than the original liver homogenate, which had been homogenized in an Atomix.⁷⁹

TABLE 8
THE METABOLIC FATE OF TRIIODOTHYRONINE

	Urine	Muscle	Kidney
<i>In vivo</i> Normal man	I ⁻		
Rat	I, glucuronidase	TRIAC I ⁻ 3,3'-T ₂	TRIAC 3,3'-T ₂
<i>In vitro</i> Liver slice Perfused rabbit liver Kidney solubilized mitochondria Liver solubilized mitochondria Liver "extract" Brain homogenate Brain homogenate (Hg ⁺⁺) Muscle Snake venom	I ⁻ (slowly) I ⁻ TRIAC TRIAC I ⁻ I ⁻ , TRIAC TRIAC I ⁻ TRIAC		

TABLE 9
THE METABOLIC FATE OF VARIOUS IODOTHYROACIDS

Substrate		Product
3,3',5'-T ₃	Rat <i>in vivo</i> Normal man	3,3',5'-T ₃ , 3,3'-T ₂ 3,3'-T ₂ -acetate in kidney I ⁻ in urine
3,3'-T ₂	Rat <i>in vivo</i> Rat <i>in vivo</i> Normal man	I ⁻ in urine I ⁻ in urine, acetate in kidney I ⁻ in urine
TETRAC	Rat muscle Rat <i>in vivo</i>	I ⁻ (slowly) I ⁻ in urine
TRIAC	Rat muscle Rat <i>in vivo</i>	I ⁻ (slowly) I ⁻ in urine
TETRAPROP	Rat muscle	I ⁻
TRIPROP	Rat muscle	I ⁻

Both T₄ and T₃ were deiodinated by the system. The product was iodide. Only minute amounts of T₃ were found if, indeed, any was demonstrated with certainty. TETRAC and TRIAC were not identified. I¹³¹-labeled TETRAC and TRIAC were deiodinated much less rapidly and, accordingly, their failure to appear upon incubation of T₄ or T₃ eliminates TETRAC and TRIAC as

intermediates in deiodination by this system. A similar deiodination was reported in spleen, kidney, muscle, adrenal and, detectably, in thyroid. Liver was most active. The system was inhibited by anaerobiosis, $10^{-1} M$ CN^{-} , citrate, and somewhat by 4-hydroxy-3:5-diiodobenzoate, and $10^{-1} M$ iodide. Citrate inhibition was reversed by ferric ion.

Relative heat stability of a deiodinating system of the liver has been confirmed by others. Etling and Barker⁷⁷ found that heat-killed liver slices incubated for 3 days with T_4 deiodinated about as well as unheated samples. Plaskett, using T_4 labeled in the 3 position, found a substance hydrolyzable to DIT as a product.⁸⁹ Lissitzky *et al.* found deiodinating activity in homogenates that had been heated for as long as 2 hours.⁹⁶ They found that deiodination continued even when the system had been deproteinized with 70 per cent alcohol and NH_3 .

Understandably, the heat-resistant deiodinating system has been challenged on several grounds, including that of its enzymatic nature, its biological relevance, and the possibility that the phenomena are artifacts akin to one described by Tata^{9,10} wherein transient alterations in the iodothyronine molecule at the time of dilution from an aqueous organic solvent cause the molecule to have chromatographic characteristics leading to its confusion with iodide. Studies from this laboratory extending over nearly two years may aid in clarifying to a degree these problems and perhaps will suggest experiments that may point toward an understanding of the processes involved.

Little deiodination is observed within 20 min. to 1 hour after adding T_4 to a whole diluted liver homogenate. If the homogenate has been heated for 2 min. at $100^{\circ} C$. by immersion in a boiling water bath and is resuspended prior to the addition of labeled T_4 , a considerable increase in deiodination is recorded as compared to unheated controls. The results of a typical experiment may be seen in TABLE 10. Virtually no deiodination occurs in either the heated or unheated soluble fraction prepared by centrifuging at $105,000 \times g$ for 1 hour. Microsomes alone fail to deiodinate and, when preheated and resuspended, there is little deiodination, providing they are washed and resuspended before heating. Addition of heated microsomes to unheated supernatant gives excellent deiodination and, when both are preheated, maximal deiodination is achieved. When the preparation is preheated at lower temperatures or at $100^{\circ} C$. for much longer periods of time deiodination is reduced. Deiodination is optimal at $37^{\circ} C$. and is reduced at lower and higher temperatures.

When it was found that an active preparation retained its activity after acidification to pH 1 and reneutralization but not when it was made temporarily alkaline, it seemed possible that an alkali-labile factor was present in the soluble fraction and was needed for the activity of the washed, heated microsomes. Accordingly, the effects of cysteine, reduced glutathione (GSH), and ascorbate were tried. Typical results appear in TABLE 11. Cysteine or GSH stimulates unheated microsomes, but heated microsomes are stimulated much more. Ascorbate is less active than reduced GSH or cysteine.

It was observed early that EDTA inhibits deiodination in the heated preparation. Accordingly, it seemed probable that a metal is involved in the deiodination process. It was then found that a heated whole homogenate preparation dialyzed for several hours against $5 \times 10^{-4} M$ Versene and then against two

changes of Tris buffer was entirely inactive when 10^{-3} *M* cysteine was present. To this system a series of metals was added to give a final metal concentration of 10^{-4} . All the physiological metals were tested, including Fe^{+++} . Only Fe^{++}

TABLE 10
EFFECT OF PREHEATING ON DEIODINATION OF THYROXINE*

	Per cent deiodination
Liver	2.6
Liver, preheated	33.2
Kidney	0
Kidney, preheated	29.2

* Each flask contained an amount of a 12,000 g supernatant of a homogenate equivalent to 150 mg. whole sheep tissue, 5 μg . I^{131} -labeled L-T_4 , thiouracil 10^{-3} *M*, 0.1 *M* Tris buffer pH 7.4 to a total volume of 4 ml., and I^{131} -labeled T_4 . Heated tissues were immersed in a boiling-water bath for 2 min. Total volume, 4 ml. Incubation for 20 min. at 37°C . Duplicate flasks.

TABLE 11
EFFECT OF REDUCED GLUTATHIONE (GSH) ON DEIODINATION*

	Per cent deiodination
Washed microsomes	3.6
Washed microsomes + GSH 10^{-3} <i>M</i>	9.2
Washed microsomes, preheated	5.3
Washed microsomes, preheated, GSH 10^{-3} <i>M</i>	33.6

* The requirement for GSH for T_4 deiodination. Each flask contained the equivalent of 150 mg. wet rat liver, 5 mg. of L-T_4 , I^{131} -labeled L-T_4 , and Tris buffer at pH 7.1. Total volume, 4 ml. Incubation time, 20 min. at 37°C . Triplicate flasks.

TABLE 12
REQUIREMENT FOR Fe^{++} FOR THYROXINE DEIODINATION*

	Per cent deiodination
Versene-dialyzed homogenate	0
Versene-dialyzed homogenate + Fe^{++} 10^{-4} <i>M</i>	0
Versene-dialyzed homogenate + cysteine 10^{-3} <i>M</i>	0
Versene-dialyzed homogenate + Fe^{++} + cysteine	39.3

* Each flask contained an amount of 12,000 g supernatant from a sheep liver homogenate equivalent to 150 mg. whole liver, thiouracil 10^{-3} *M*, 5 μg . L-T_4 , and I^{131} -labeled L-T_4 . Total volume to 4 ml. with 0.1 *M* Tris buffer pH 7.3. Dialysis was overnight against 0.1 *M* phosphate buffer containing 5×10^{-4} *M* Versene, followed by two changes of buffer without Versene for 2 hours each. Duplicate flasks.

proved effective (TABLE 12). It should be noted that cysteine and Fe^{++} in the same or varied concentrations over the same time range but without homogenate gave little or no deiodination of T_4 . The precise concentration relationships between Fe^{++} and cysteine have not been worked out for optimal deiodination, but Fe^{++} is inhibitory in concentrations much above 10^{-4} *M*.

A number of other properties of the system have been recorded. It is in-

hibited by small quantities of fresh serum, but not by boiled serum. Phosphate in addition to that in the washed microsomes is not required. Oxygen is required. Incubation of the heated microsomes for 4 hours at pH 8.2 with crude trypsin before returning to pH 7.0 is completely inhibitory, whereas this pH change and incubation does not inhibit without added trypsin, and added trypsin without incubation does not inhibit (TABLE 13). Parachloromercuribenzoate at 10^{-4} M is inhibitory, and so are 10^{-2} CN⁻ and penicillamine. Citrate and pyrophosphate inhibit. The microsomes may be precipitated with 8 per cent final concentration perchloric acid and then reconstituted at neutral pH, with excellent retention of deiodinating capacity. Deiodination is not dependent upon the presence of light. It proceeds with or without a small amount of crystalline albumin in the medium.

Evidence for a catalytic reaction, as opposed to a stoichiometric one, has been sought in studies of the effect of substrate concentration on reaction rate.

TABLE 13
EFFECT OF TRYPSIN ON THYROXINE DEIODINATION*

	Per cent deiodination
Tissue alone	0.3
Tissue + GSH 10^{-3} M	38.1
Tissue, preincubated with trypsin, + GSH	11.8
Tissue, preincubated with trypsin, no GSH	0.3
Tissue + trypsin without preincubation + GSH	31.6

* Effect of trypsin on T₄ deiodination. The rat liver microsomes were prepared by an 8% final concentration perchloric acid precipitation, followed by neutralization and resuspension. Each flask contained the equivalent of 150 mg. wet rat liver tissue. Pretreatment with 10 mg. crude trypsin was for 4 hours and readjustment of the pH to 7.1. The bottom set of tubes was incubated for 4 hours at pH 8.2 without trypsin before readjustment to pH 7.1. Incubation time, 20 min. Triplicate flasks.

A set of results appears in FIGURE 1. It is seen that the reaction is that of a catalytic rather than a stoichiometric process. Although the deiodination is not strictly linear during the 20 min. of incubation, it is sufficiently nearly so for purposes of interpretation of FIGURE 1. A timed curve of deiodination under identical conditions appears in FIGURE 2. In this experiment, as in others, the fraction deiodinated is calculated as the difference from paired flasks differing only in that buffer was substituted for homogenate. Actually, the controls, even after 120 min., had only a small percentage more iodide than was present initially in the labeled T₄ solution. The 10^{-3} M thiouracil appears to contribute to this stability.

Deiodination by this system appears to depend upon the presence of oxygen and ferrous ion and, in addition, is much potentiated by reducing substances such as cysteine, reduced GSH, or ascorbate. One of the noteworthy facts is the potentiation by heating. It has been noted by Lardy⁹⁵ that heating of a kidney homogenate opens up sulfhydryl groups, but this alone cannot be the explanation for the effect of heat, because stimulation of unheated microsomes by 10^{-3} M GSH is decidedly submaximal.

Other explanations for the activating effect of heat include a destruction of

a sulfhydryl oxidizing system that, in the unheated preparation, oxidizes the needed groups. If this were the case, one would expect that incubation of an unheated homogenate before heating would permit oxidation of SH^- groups and, accordingly, would abolish the stimulating effect of heat. This did not occur. Heat activation also could be, in fact, destruction of a binding protein for T_4 ; however, washed microsomes bind T_4 very poorly. No other satisfactory explanation for the phenomenon is apparent.

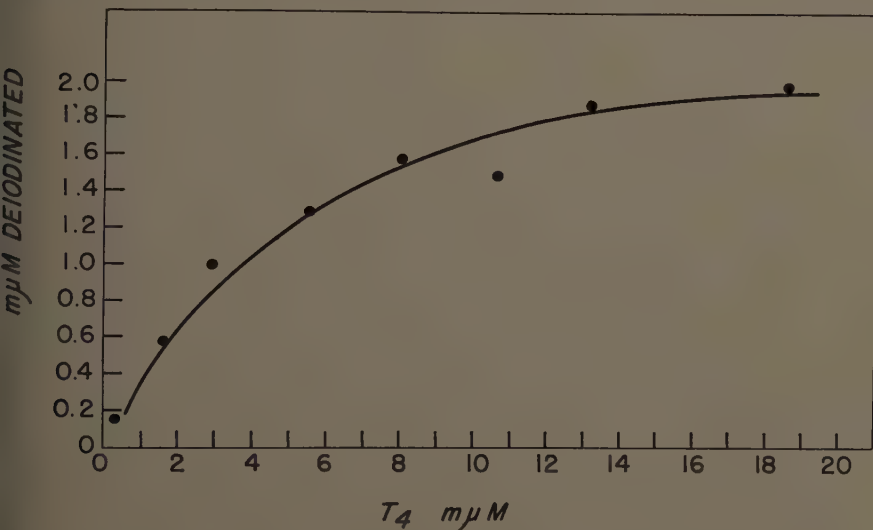


FIGURE 1. The deiodinization of T_4 as influenced by substrate concentrations. Each flask contained heated microsomes equivalent to 150 mg. of whole rat liver, 1^{31} -labeled L-T_4 , $10^{-3} M$ thiouracil, $10^{-3} M$ GSH, and Tris buffer to a total volume of 4 ml. pH 7.1. Incubation time, 20 min.

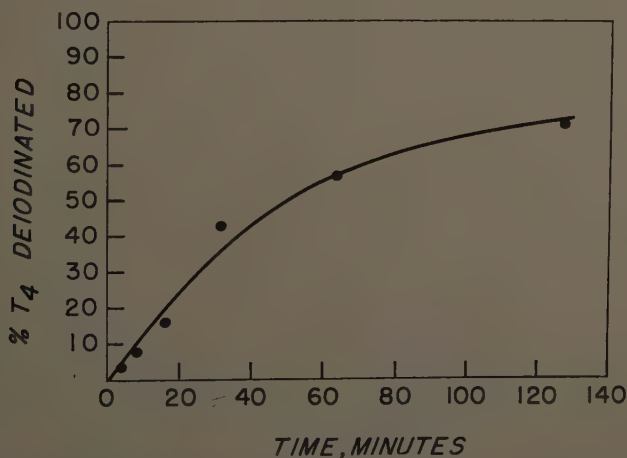


FIGURE 2. Deiodinization of T_4 by a heated liver microsomal preparation. Flask contents as in FIGURE 1, except $5 \mu\text{g. L-T}_4$ per flask.

The problem of the enzymatic nature of the reaction is an important one. It has been shown that the factor contributed by the microsomes acts catalytically, that it is not dialyzable, that it is lost after treatment with crude trypsin, which in itself is not inhibitory, that it has a temperature optimum of 37°C ., and that it follows protein through perchloric acid and alcohol precipitations. I have not succeeded in solubilizing it by any method, even by butanol extraction, sonic treatment, and desoxycholate treatment with subsequent dialysis. My inclination at present, therefore, is to consider the process an enzymatic one. Resistance to heat and to alcohol precipitation has ample precedent among other enzymes, and in itself offers no compelling argument against an enzymatic nature of the observed deiodination.

An important issue is whether in these experiments with the heated liver homogenate the "deiodination" described by Tata^{9,10} has been avoided. It appears to have been, for several reasons. The labeled T_4 was always added to the incubating flask from a dilute aqueous solution. Albumin and microsomal protein were present. The iodide formed was shown to be iodide in several chromatographic systems and by extraction with CS_2 , after equilibration with KIO_3 . The deiodination was progressive with the time of incubation: there was not the slow reversal found by Tata.^{9,10} Finally, deiodination was not appreciable in control incubation flasks.

What, then, is the nature of the deiodination reaction? This question cannot be answered at present. A large number of aromatic ring-splitting enzymes that require oxygen, ferrous ion, and cysteine or reduced GSH are now well described.⁹⁷⁻¹⁰⁰ Several of these oxidize a hydroxy group ortho to a hydroxy or carboxy group, and break the ring. Among these is catechol oxidase. By analogy one might speculate that the deiodination reaction is a hydroxylation and sequential oxidation at the 3' position, ring rupture, and splitting of the oxygen bridge. This interpretation is consistent with the finding of Plaskett⁸⁹ that DIT can be obtained easily by deiodination. This explanation is also consistent with the failure of most workers to find significant amounts of iodothyronines in *in vitro* deiodinating systems, although iodide appears often in large amounts.

Deiodination by tissue-free systems containing Fe^{++} , ascorbate, oxygen, EDTA, and various substrates of the iodotyrosine and iodothyronine series and related compounds has been studied extensively by Lissitzky and Roques.^{5,101} Udenfriend⁶ had shown earlier that such a system at neutral pH and at 37°C . can hydroxylate many aromatic compounds, including tyrosine and phenylalanine,¹⁰² and Michaelis¹⁰³ long ago constructed an autocatalytic complex of cysteine and Fe^{++} that produced peroxide. When MIT was incubated for several hours in Lissitzky's system, iodide, tyrosine, and 3:4-dihydroxyphenylalanine were found, together with 2 other iodinated compounds, one of which was almost certainly 3-iodo-4:5-hydroxyphenylalanine. DIT was attacked poorly by the system; only small amounts of MIT, I^- , and the 5-OH-MIT appeared. Evidence of ring rupture, deiodination, and phenolic ring hydroxylation was obtained when the iodinated thyronines were studied; however, thyroxine was very poorly attacked.

On the other hand, a Cu^{++} -bis-quinoline system failed to attack T_3 , but

attacked T_4 to give I^- , DIT, a small amount of T_3 , and an unidentified substance.¹⁰¹

It remains to be learned whether the Fe^{++} -ascorbate-oxygen system and the horseradish peroxidase¹⁰⁴ and polyphenol oxidase¹⁰⁵ systems are precise models for the deiodination effected by the heat-stable liver system or the Fe^{++} -requiring muscle system described by Tata.⁹⁴ One cannot escape the inference that the liver homogenate catalyzes an analogous group of reactions. Present evidence suggests that the principal difference is one of rate.

Summary

Mono- and diiodotyrosine are deiodinated by a heat-labile microsomal enzyme that requires TPNH. The deiodinase is found in thyroid, liver, kidney, and elsewhere.

Pathways of deiodination of the iodothyronines and related compounds are present in several organs. Deiodination of T_4 to T_3 occurs in kidney slices, but disappears with homogenization. Deiodination also occurs in brain, liver, and muscle homogenate preparations, but the products of this deiodination are not well established. Except for the kidney slice preparation of Albright *et al.*,⁷² there is at present surprisingly limited evidence for T_3 as a principal deiodination product of T_4 , either *in vivo* or in organ slices or homogenates.

The heat-stable T_4 -deiodinating system of liver is heat-activated and requires ferrous ion, oxygen, and reduced glutathione, ascorbate, or cysteine. Evidence has been presented that this system is catalytic in nature. It is inferred that uptake of the phenolic ring accompanies the deiodination.

The T_4 deiodinase of brain and muscle is different in several particulars from the heat-stable liver system. It is principally in the soluble fraction of the homogenate preparation. Oxidative deamination is not a requisite for this deiodination.

Several interesting model systems, some employing ferrous ion, ascorbate, and oxygen, and others employing horseradish peroxidase and polyphenoloxidase have been employed in the study of the degradation of the iodoamino acids. The relevance of these to the problem of tissue deiodination remains to be clarified.

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Part II. Transport and Metabolism of Thyroid Hormones

THE INTERACTION OF THE THYROID HORMONES WITH THE PROTEINS OF HUMAN PLASMA*

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Seven years ago Gordon *et al.*¹ first described the association of thyroxine† with an alpha globulin moiety during electrophoresis of serum on filter paper in Veronal buffer, pH 8.6. It was soon clear that in this system thyroxine was associated principally with a protein migrating in the zone between the α_1 - and α_2 -globulins.²⁻⁴ This came to be known as the thyroxine-binding protein (TBP) or thyroxine-binding globulin (TBG). Although it had long been known that the thyroid hormone participates in an interaction with plasma protein that renders it protein-precipitable⁵⁻⁸ and nonultrafilterable,⁹ demonstration of the specificity of this interaction thus depended and continues to depend largely on electrophoretic techniques. Many studies of the interaction of thyroxine and other iodinated materials with the proteins of whole and fractionated plasma quickly followed.¹⁰⁻¹⁵ Of these, the great majority employed paper electrophoresis of proteins in Veronal buffer, pH 8.6. For a time a complacent view of the rapidly expanding body of data seemed justified. However, within the past 2 years new information has markedly complicated the interpretation of earlier findings. It is the purpose of this communication to describe these recent findings and to attempt to resolve certain of the interpretative difficulties that have arisen from them.

Sources of present confusion can be understood only in the light of the voluminous data obtained in earlier studies. These data have been reviewed extensively elsewhere¹⁶⁻¹⁸ and are therefore considered here only briefly.

A variety of studies has demonstrated that endogenous thyroxine in serum and that added exogenously are readily and completely exchangeable among the thyroxine-binding proteins.^{15,16} Thus, for any electrophoretic system, the distribution of exogenous labeled thyroxine probable reflects quite accurately the distribution of the endogenous hormone within that system. When serum is enriched with quantities of I¹³¹-labeled thyroxine so small that the total hormonal concentration is not appreciably increased, paper electrophoresis in Veronal buffer, pH 8.6, shows that approximately 90 per cent of the hormone is bound by TBG, while the remainder is associated with albumin. As serum is enriched with increasing concentrations of stable hormone, there occurs a progressive displacement of labeled hormone from TBG onto albumin, so that an increasing percentage of thyroxine is associated with albumin and a corre-

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† Unless otherwise specified, the terms for the iodinated amino acids refer to the levorotatory isomers.

spondingly smaller percentage with TBG. Nevertheless, calculated values for the total quantity of thyroxine associated with TBG (total hormonal concentration \times per cent bound to TBG) increase continuously as hormonal concentration rises. This failure to demonstrate a binding maximum for TBG during electrophoresis performed in the traditional manner was ascribed to trailing of albumin carrying with it thyroxine across the TBG zone.¹² Robbins¹⁹ therefore devised a system of reverse-flow electrophoresis in which hydrodynamic flow opposes the electrophoretic migration of albumin and therefore diminishes trailing of this component. Employing this technique in Veronal buffer, he found the average thyroxine-binding capacity for TBG in normal serum to be approximately 20 $\mu\text{g.}/100\text{ ml.}$ ¹⁹ The thyroxine-binding capacity of albumin appeared to be virtually unlimited. By means of this and related techniques, alterations in the thyroxine-binding activity of TBG were noted in the sera of patients with a number of abnormal states.^{16,20-23}

The importance of obtaining TBG in purified form was recognized early. Initial steps in this effort demonstrated that TBG was highly concentrated in certain fractions of the plasma proteins prepared by Method 6 of Cohn and his colleagues, especially Fraction IV-6.¹⁵ It had also been noted that the isoelectric point of TBG was less than 4.5, as indicated by its anodal migration at this pH .¹⁴ The acidic character of this protein, together with its concentration in specific plasma fractions, was employed in initial efforts to isolate TBG. Ion-exchange chromatography on Dowex I equilibrated with acetate buffer, pH 4.5, effectuated a good separation of TBG from the bulk of other proteins in Cohn fractions, but resulted in variable loss of its thyroxine-binding potency.²⁴ This technique was therefore considered unsatisfactory.

Thus, by mid-1956 it appeared that we were rapidly approaching a complete and accurate understanding of the quantitative and qualitative aspects of the interactions between thyroxine and other thyroid hormones with the plasma proteins. In addition, a hopeful start had been made toward the isolation of TBG itself. Since that time, however, a variety of new data has been accumulated that tends to obscure the significance of earlier findings and has introduced newly recognized variables into the field. These findings can be understood best by considering events in their chronological sequence.

During portions of 1956 and 1957 I continued my efforts to isolate TBG in the laboratory of T. S. Work at the National Institute for Medical Research, London, England. There, after numerous abortive attempts, a technique was ultimately devised that made possible the isolation of a highly active interalpha thyroxine-binding protein that appeared to be homogeneous by electrophoretic and ultracentrifugal criteria. Cohn Fraction IV-4* was subjected to ammonium sulfate precipitation, and the resulting product adsorbed by a batch technique to the diethylaminoethyl cellulose resin of Peterson and Sober²⁵ in acetate buffer, pH 4.5. Eluates from the resin contained TBG and a nonthyroxine-binding α_2 -globulin in varying proportions. These were subsequently separated by preparative electrophoresis in cellulose columns.²⁶ However, resin eluates also contained a third protein present in sufficient concentration to stain with bromphenol blue, which appeared to bind thyroxine avidly during

* This material was kindly supplied by John M. Newell and Roderick C. Dwyer of the Massachusetts Public Health Biologic Laboratories, Boston, Mass.

paper electrophoresis in Veronal buffer (FIGURE 1). This protein, too, was readily separable from other components of the resin eluate by column electrophoresis. The rapid anodal migration of this component was similar to that of a prealbumin whose isolation from serum had been described the year before by Schultze *et al.*²⁷ and to that of the prealbumins demonstrable in serum during starch-gel²⁸ and free electrophoresis.²⁹ Because of possible differences between this prealbumin and those demonstrated by other workers, which had not been shown to bind thyroxine, the former has been designated the thyroxine-binding prealbumin or TBPA. Although binding of thyroxine by a prealbumin had occasionally been noted in abnormal sera¹⁶ and other biological fluids electrophoresed in Veronal buffer,³⁰ its appearance in normal serum was so uncommon that it was generally overlooked. The emergence of a purified prealbumin as an avid binder of thyroxine therefore was unexpected; we had been conditioned to think of TBG as the sole primary thyroxine-binding protein. It therefore seemed possible that the apparent binding of thyroxine by TBPA might be merely an electrophoretic artifact. Furthermore, since many serum proteins are capable of binding thyroxine to at least a limited extent,¹⁵ it became important to determine the relative intensity of the thyroxine-TBPA bond. Solutions of purified prealbumin were enriched with equal concentrations of human serum albumin, I¹³¹-labeled thyroxine, and varying concentrations of stable thyroxine. Electrophoresis of these mixtures in Veronal buffer, pH 8.6, revealed the fact that over a wide range of concentrations thyroxine was bound predominantly by prealbumin. This finding indicated that the thyroxine was indeed bound to prealbumin and that the intensity of the interaction between thyroxine and this protein was far greater than that between thyroxine and albumin. Further evidence of the strong association between thyroxine and prealbumin was obtained by a nonelectrophoretic technique. Prealbumin was found to inhibit strongly the spontaneous deiodination of thyroxine *in vitro*.^{*} This has been considered an indication of an intense protein-hormone interaction.³¹

At this point, it seemed possible that prealbumin might represent merely an artifact of fractionation procedures and might not be present in unfractionated normal plasma. Some evidence for this had been obtained during the purification of TBG. In the preparation of cellulose columns it was customary to test the adequacy of the column by the electrophoresis thereon of normal serum to which labeled thyroxine had been added. Because of the good electrophoretic resolution that it appeared to afford, a buffer system containing trishydroxymethylaminomethane (Tris) and maleic acid was recommended by Rodney R. Porter. When sera were electrophoresed in cellulose columns in this buffer system, TBPA could regularly be demonstrated. Accordingly, experiments were performed to determine whether demonstration of TBPA in serum depended upon this buffer system or upon other conditions of column electrophoresis. Findings in these experiments, reported in brief elsewhere,³² have been misinterpreted by recent authors,^{33,34} who have suggested that TBPA could be demonstrated in serum only when serum had been supplemented with fractions rich in this protein. This was not the case. Indeed, it

* This test was kindly performed by Jamshed R. Tata.

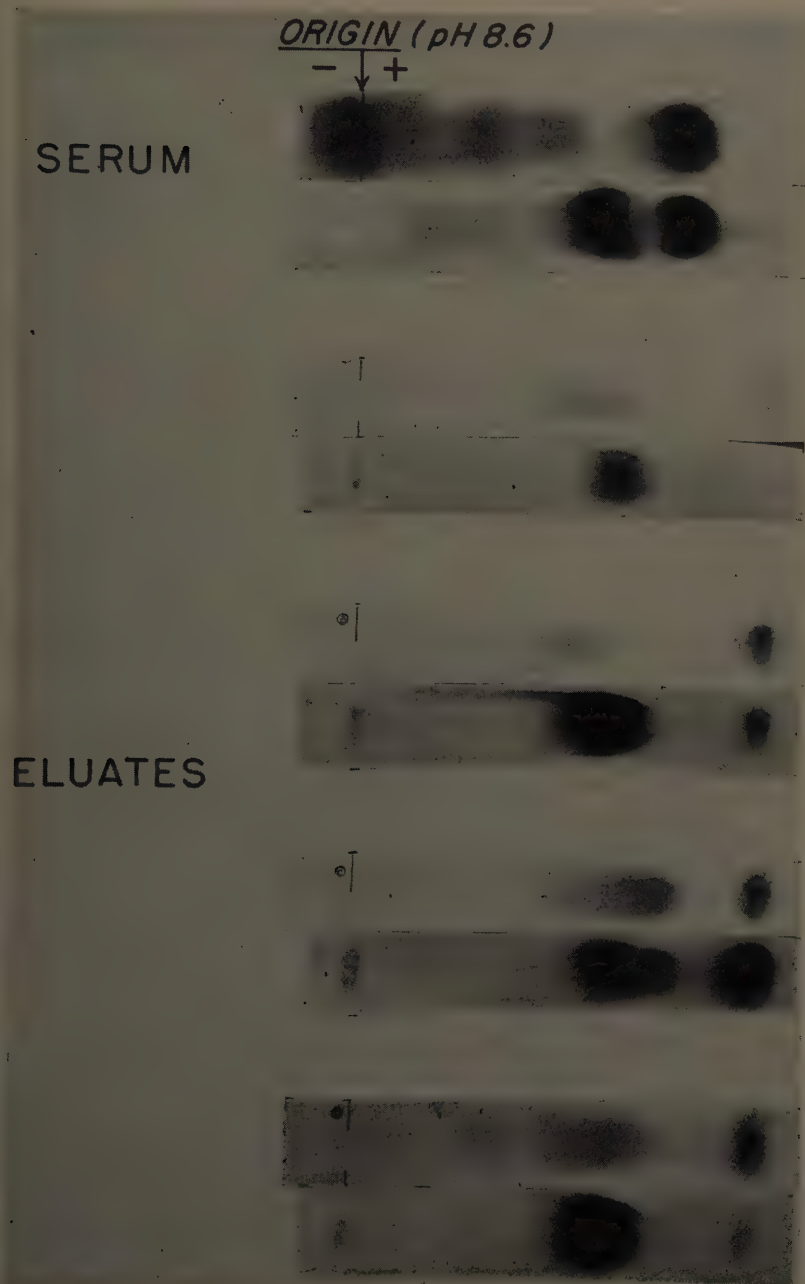


FIGURE 1. Proteins eluted from diethylaminoethyl cellulose resin after equilibration with ammonium sulfate fraction of Cohn Fraction IV-4 in acetate buffer, pH 4.5. In each pair, bromphenol blue stain of electrophoretic strip is shown above, and radioautograph for localization of added labeled thyroxine is shown below. Electrophoresis on filter paper in Veronal buffer, 0.06 M, pH 8.6. Concurrent electrophoresis of serum shown in upper pair as reference.

was reported that TBPA could be demonstrated during both column and paper electrophoresis of unfractionated, unenriched normal serum, provided that Tris-maleate, rather than Veronal, was employed as the buffer system.

These differences between findings obtained during electrophoresis of serum in Veronal and in Tris-maleate buffers form the nucleus of current conflicts of opinion with regard to the thyroid hormone-plasma protein bond. Furthermore, they emphasize the hazards of interpreting electrophoretic data. During electrophoresis, exposure of interactants to strong electric fields and to abnormal conditions of pH or ionic strength and composition, as well as possible interactions between protein or hormone and the supporting medium or buffer anions, may obscure conditions that truly pertain *in vivo*. In the present discrepancy ionic composition appears to be the most important variable. Thus, it seems possible that Veronal might interfere with the binding of thyroxine by TBPA or might promote an interaction between TBPA and other proteins, so that the electrophoretic mobility and identity of the former would be altered. On the other hand, TBPA might be an artifact induced by electrophoresis in Tris-maleate buffer. These anions might induce or facilitate the binding of thyroxine by a prealbumin with no intrinsic binding capacity of its own. Tata³³ has recently presented data that lead to an alternate suggestion, namely that TBG is the result of an interaction between TBPA and another serum protein. He suggested that in the circulation the greatest portion of the thyroxine-binding prealbumin is present in this interacted form. This hypothesis would imply that the Tris-maleate system prevents or dissociates this protein-protein interaction.

It is apparent that no discussion of the interaction of the thyroid hormones with the proteins of plasma can proceed without attempting to resolve the dilemma posed by the foregoing conflicting findings. Basically, two questions require consideration: Is the binding of thyroxine by a serum prealbumin artifactually induced by the Tris-maleate buffer? Is TBPA, in essence, a fragment dissociated during electrophoresis in Tris-maleate buffer from a protein-protein complex that is TBG? The present discussion will attempt to answer these questions and, in so doing, will describe many features of the thyroid hormone-plasma protein interaction as revealed by revised electrophoretic and other techniques.

Is the Tris-maleate buffer system necessary for the demonstration of thyroxine-binding by a human serum prealbumin? Paper electrophoretic demonstration of TBPA does not require previous or current exposure of serum protein to Tris-maleate buffer. Samples of prealbumin prepared by the method described above, as well as by the method of Schultze and his co-workers, avidly bind thyroxine, but have not been exposed to the Tris-maleate system.³³ However, it remains possible that fractionation procedures themselves may alter serum proteins in such a way as to lead to the artifactual formation of TBPA.

This seems unlikely in view of the fact that we have found TBPA in human serum during paper electrophoresis, not only in Tris-maleate buffer, but also in borate, phosphate, alanine and, as Beierwaltes and Robbins³⁵ have recently reported, in carbonate buffer. If Tris-maleate introduces qualitative artifacts in hormonal binding, then this property must be shared by several other organic and inorganic anions.

TBPA has been occasionally noted in nephrotic sera³⁶ and is regularly seen in cerebrospinal fluid during paper electrophoresis in Veronal buffer.³⁰ Both TBG and TBPA are found to bind thyroxine prominently when normal serum is subjected to continuous curtain electrophoresis in the Durrum system.³⁷

Finally, derivatives of thyroxine such as tetraiodothyroacetic acid (TETRAC), which bind to TBPA more firmly than does thyroxine, regularly migrate in association with TBPA during paper electrophoresis of normal serum in Veronal buffer (*see below*).

Aly and Niederhellmann³⁸ have reported that, when large rather than small aliquots of serum are applied to filter paper, prealbumin is readily demonstrable by bromphenol blue stain following electrophoresis in Veronal buffer. These authors suggested that the rapid migration of prealbumin makes it particularly susceptible to adsorption to filter paper during electrophoresis. By virtue of the saturation of binding sites along the electrophoretic path, larger quantities of prealbumin might thus achieve their true electrophoretic migration. While the prealbumin noted by Aly and Niederhellmann has not been shown to bind thyroxine, it is thought to be identical with that prepared by Schultze *et al.*²⁷ The latter protein binds thyroxine avidly, as noted below.

The foregoing observations indicate that when the protein is present in sufficient concentration or is allowed to interact with a hormone for which its avidity is sufficiently intense, both its characteristic migration and hormonal binding propensity are demonstrable in Veronal buffer. Tris-maleate and other buffer systems would therefore appear to make possible the demonstration of these properties at the lower concentration and lesser binding affinities that obtain in normal serum containing labeled thyroxine.

Thyroxine-binding capacities of TBG and TBPA in normal and abnormal sera. In the Tris-maleate system and in the bicarbonate system it is possible to assess binding maxima for TBG and TBPA without recourse to reverse-flow electrophoresis. In the former buffer, normal values average 22 $\mu\text{g. thyroxine/100 ml. of normal serum}$ for TBG and 120 $\mu\text{g./100 ml. for TBPA}$. The former value is virtually identical with that found by Robbins¹⁹ for TBG employing Veronal buffer in the reverse-flow system. If Tris-maleate and other buffers make possible the demonstration of TBPA by dissociating active binding sites from TBG, then the binding capacity of TBG in such systems would be expected to be less than in Veronal. Evidently, such is not the case.

Further evidence of the independence of TBG and TBPA is provided by studies performed in a patient similar to one recently described by Tanaka and Starr.³⁹ Despite a PBI of 2.0 $\mu\text{g. per cent}$, the present patient displayed neither clinical nor other laboratory evidence of hypothyroidism. When the patient's serum was electrophoresed in Tris-maleate buffer, labeled thyroxine bound to TBPA and to albumin, but significant binding to TBG could not be discerned, although the concentration of added stable thyroxine was varied over a range from 0.2 to 458 $\mu\text{g. per cent}$ (FIGURE 2). In Veronal buffer binding to TBPA was not seen, and albumin appeared to be the principal thyroxine-binding component. Despite the virtually complete absence of TBG, the thyroxine-binding capacity of TBPA was normal. In this patient's serum, therefore, normal quantities of TBPA could not have arisen from the dissociation of TBG, since appreciable quantities of the latter were not present. It might be postu-

lated that this patient's serum lacked the hypothetical protein with which TBPA interacts. This explanation, however, does not account for the failure to demonstrate TBPA during electrophoresis of his serum in Veronal buffer.

During the past 18 months binding capacities of TBG and TBPA have been assessed in approximately 300 patients with a variety of abnormal states. Independent variations in the binding capacities of these proteins have provided further evidence that they are probably distinct from one another. For

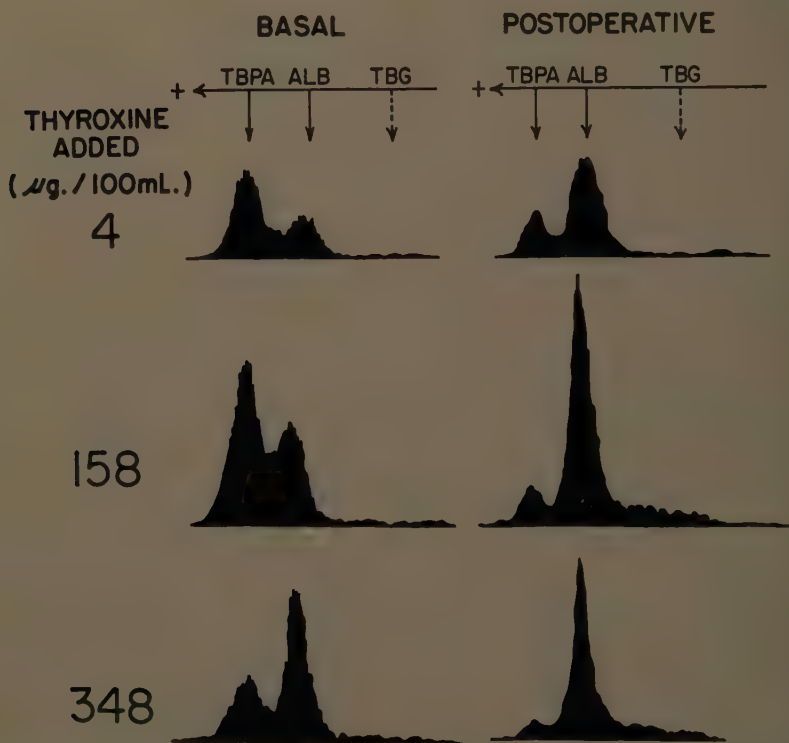


FIGURE 2. Effect of inguinal herniorrhaphy on the binding of thyroxine in the serum of a patient with decreased TBG. Scans of the localization of added labeled thyroxine. Postoperative serum obtained 48 hours after surgery. Electrophoresis on filter paper in Tris-maleate buffer, pH 8.6.

example, the marked increase in TBG that occurs during normal pregnancy^{20,21} is not associated with changes in the binding capacity of TBPA. Furthermore, although the binding of thyroxine by TBPA abruptly declines *post partum*, the increased binding capacity of TBG persists. Recent studies have also shown that the thyroxine-binding capacity of TBPA is often but not always diminished in patients with active thyrotoxicosis and in patients with a variety of nonthyroidal illnesses, including febrile and postoperative states, malignancy, cerebral and myocardial infarction, and cirrhosis.⁴⁰ Despite abnormalities in TBPA, the binding capacity of TBG in such patients is not significantly different from normal. Similar changes in the binding capacity of TBPA were

noted following inguinal herniorrhaphy in the patient described above, in whom TBG was virtually absent. This finding indicates that the coexistence of neither TBG nor the hypothetical TBPA-binding protein of which it may be composed is necessary for the decrease in thyroxine-binding by TBPA that occurs during nonspecific illness.

The distribution of TBG and TBPA in plasma fractions. If TBPA were a fragment of a protein-protein complex comprising TBG but dissociated therefrom by buffers other than Veronal, then, in such buffer systems TBPA should be present whenever TBG is present. An analysis of the distribution of the 2 proteins among Cohn fractions of plasma indicates that this is not the case. Experiments were performed with Cohn Fractions IV-4, IV-5, IV-6, IV-7, IV-8, and IV-9. All solutions were adjusted to a constant protein concentration of 3.0 gm. per cent. Aliquots of each were enriched with an equal concentration of purified human serum albumin. The specimens were then subdivided once again and were labeled with radioactive thyroxine of either high or low specific activity. Samples were then electrophoresed concurrently in Tris-maleate buffer. This technique permits the identification of small quantities of binding proteins even when these are accompanied by high concentrations of proteins of more intense thyroxine-binding avidity. TBG was clearly seen in all fractions. However, TBPA was absent from fractions IV-7, IV-8, and IV-9. In other fractions no correlation, either inverse or direct, between the binding activity of TBG and that of TBPA could be demonstrated.

Purified preparations of TBG have been subjected to repeated electrophoresis on cellulose columns in Tris-maleate buffer. In no instance have appreciable quantities of TBPA been dissociated therefrom.

The relative affinities of TBG and TBPA for L-thyroxine. If TBG were a complex of TBPA and an α -globulin with no thyroxine-binding potency of its own, then the thyroxine-binding avidity per unit weight of TBPA should exceed that of TBG. Tata³¹ has reported that, as adjudged by its ability to inhibit the spontaneous deiodination of thyroxine *in vitro*, prealbumin prepared by Schultze and his coworkers was the most potent protein tested. However, tests were apparently not performed with purified TBG.

While absolute thyroxine-binding constants for TBG and TBPA have not as yet been determined, equilibrium dialysis experiments performed in my laboratory indicate that, per unit weight, the binding affinity of highly refined TBG exceeds that of TBPA by severalfold.

The binding of thyroxine congeners. Further evidence of striking differences between TBG and TBPA is afforded by studies of their relative binding affinity for several congeners of thyroxine. While L-thyroxine binds avidly to both TBG and TBPA, as well as to albumin, saturation studies in the Tris-maleate system have failed to reveal significant binding of triiodothyronine to TBPA. Here, as in Veronal buffer,^{11,13} labeled L-triiodothyronine is much more readily displaced from TBG onto albumin by the addition of either stable L-triiodothyronine or L-thyroxine than is labeled L-thyroxine itself.

Profound alterations in the binding characteristics are induced by changes in the alanine side chain of the thyronine nucleus. Here too, profound differences are noted between the binding affinities of TBG and TBPA. While L-thyroxine binds to both TBG and TBPA, its acetic and propionic acid deriva-

tives bind firmly to TBPA and secondarily to albumin, but do not bind appreciably to TBPA. Furthermore, although L-triiodothyronine binds to TBG and not to TBPA, its acetic and propionic acid analogues bind to TBPA but not to TBG. The relative intensity of binding of these analogues by TBPA has been assessed in the following manner. Sera were enriched by the addition of small quantities of labeled thyroxine or labeled derivative. Displacement

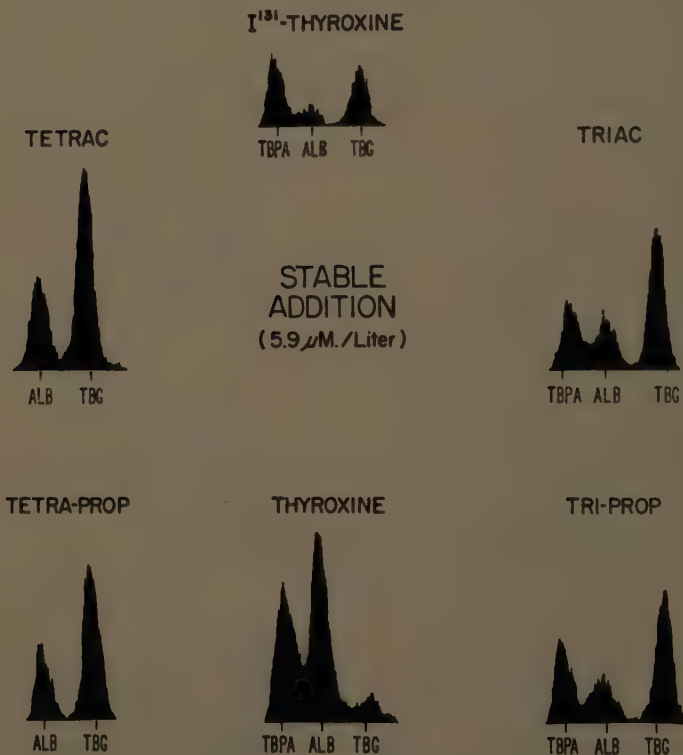


FIGURE 3. Displacement of labeled thyroxine from TBPA by the addition of stable thyroxine and its derivatives. The upper row reveals the localization of labeled thyroxine in unsupplemented serum. The lower 2 rows reveal the change in distribution of labeled thyroxine produced by supplementation of serum with 5.9 μ M./l. of individual stable iodinated compounds. Electrophoresis on filter paper in Tris-maleate buffer, pH 8.6.

of the labeled compound from TBPA onto albumin was studied following addition of increasing quantities of stable derivative or stable thyroxine. These studies indicated that the deaminated derivatives of thyroxine and triiodothyronine bind more strongly to TBPA than their amino acid analogues (FIGURE 3). However, their affinity for TBG is markedly diminished or completely lost.

Further pronounced differences between the binding affinities of TBG and TBPA were provided by studies with steric isomers of thyroxine. Sera were labeled with tracer quantities of either D- or L-thyroxine and were enriched with increasing concentrations of stable L-thyroxine. In unenriched sera some binding of D-thyroxine by TBPA was noted. However, the fraction of added

labeled hormone bound by this protein was considerably less than in the case of L-thyroxine. In sera enriched with stable L-thyroxine, labeled D-thyroxine was displaced much more readily from TBPA than labeled L-thyroxine. No difference in the displacement of the isomers from TBG could be discerned (FIGURE 4).

From the foregoing findings it may be concluded that, if TBG is indeed the result of an interaction between TBPA and another plasma protein, such inter-

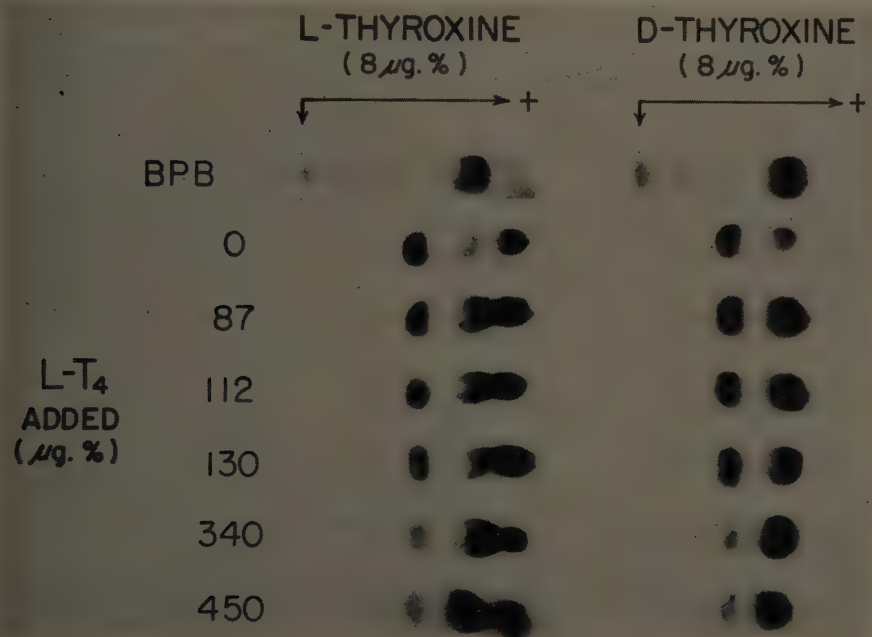


FIGURE 4. Binding of labeled isomers of thyroxine by proteins of normal human serum during electrophoresis in Tris-maleate buffer, pH 8.6. Effect of enrichment with stable L-thyroxine. BPB indicates bromphenol blue stain for localization of protein bands. Radioautographs reveal localization of labeled compounds.

actions must induce truly profound qualitative alterations in the binding properties of the prosthetic group.

The influence of Veronal. The foregoing evidence strongly indicates that TBPA is not an artifact induced by the Tris-maleate and other buffer systems, nor is it likely to be a portion of the protein-protein complex that appears as TBG. Nevertheless, it remains to be explained why the binding of thyroxine by TBPA is seen only rarely during the electrophoresis of normal serum in Veronal buffer. As noted earlier, the binding of thyroid hormones by TBPA is readily demonstrable even in Veronal buffer when the concentration of protein is sufficiently high or when the labeled hormone binds more firmly to TBPA than does thyroxine itself. These observations are consistent with the interpretation that the thyroid hormones and Veronal compete for binding sites on TBPA. Strong evidence that this is the case was obtained in the following

experiments. Normal serum was enriched with tracer quantities of labeled thyroxine and increasing quantities of stable thyroxine. Samples were then electrophoresed in Tris-maleate buffer, pH 8.6, and in Tris-maleate buffer to which 0.05 *M* Veronal had been added. In the latter system, virtually no binding of thyroxine in the prealbumin zone was seen. However, an additional band of labeled thyroxine could be detected in the α_1 -globulin area by radioautography. When sera were electrophoresed in Tris-maleate buffer containing progressively lower concentrations of Veronal, progressively increasing quantities of thyroxine appeared in the prealbumin zone. The foregoing observations might be explained if Veronal merely altered the electrophoretic migration, but not the thyroxine-binding affinity of TBPA. However, when preparations of purified prealbumin containing tracer quantities of labeled thyroxine were dialyzed against either whole serum or human serum albumin previously equilibrated with a variety of buffers, a profound inhibitory effect of Veronal on the binding of thyroxine by prealbumin was seen. It thus appears that Veronal inhibits the binding of thyroxine by TBPA, and this inhibition probably results from a competition between Veronal anions and the thyroid hormones for binding sites on the protein.

While this explanation alone would be adequate to account for the infrequent demonstration of TBPA in normal serum during electrophoresis in Veronal buffer, some evidence suggests that the action of Veronal may be more complex. In the experiments cited above, in which serum was electrophoresed in Tris-maleate buffer containing Veronal, a slight but consistent increase in the apparent binding capacity of TBG was noted in the Veronal-supplemented system. This might be ascribed to the additional α -globulin band that appeared under these conditions and would be difficult to separate from TBG by scanning procedures. Further possible evidence of an alteration in the migration of TBPA during electrophoresis in Veronal buffer was provided by studies performed in the patient whose serum displayed virtually no TBG during electrophoresis in Tris-maleate buffer. When this patient's serum was electrophoresed in Veronal buffer, virtually no binding of thyroxine by TBPA was seen. However, at low concentrations of added hormone, a small proportion of thyroxine was localized behind the albumin in the α_1 -globulin zone.

Considered together, these findings suggest that Veronal interacts with TBPA, inhibits its ability to bind thyroxine, and changes its electrophoretic mobility, possibly by altering its electrostatic charge.

Data from other laboratories. The foregoing findings suggest strongly that TBPA is not a fragment of TBG that is dissociated therefrom by the Tris-maleate buffer system. Furthermore, they appear to be adequate to explain the infrequent demonstration of TBPA in normal serum electrophoresed in Veronal buffer. However, it is necessary to consider apparently conflicting data obtained in other laboratories. Chief among these are the electrophoretic and immunological findings of Tata²³ that led him to the conclusion "that a large part of human serum prealbumin is present as a complex in the α -globulin fraction and that it is responsible for the binding of thyroxine observed in thyroxine-binding protein." These findings will be considered in brief. The tryptophan content of the prealbumin prepared by Schultze and his co-workers is said to be exceptionally high.²⁷ Tata therefore employed staining reactions

for tryptophan using Ehrlich's p-dimethylbenzaldehyde reagent,⁴¹ as a means of assessing the electrophoretic localization of prealbumin. All electrophoreses were performed in Veronal buffer. In normal serum Ehrlich-positive material was seen only in the TBG zone and was associated with the majority of added labeled thyroxine. When serum was supplemented with prealbumin prepared by Schultze a marked increase in tryptophan staining was noted in the inter-alpha area, while a less intense Ehrlich reaction together with a positive bromphenol blue stain was seen anodal to albumin. These findings were interpreted to indicate that normally prealbumin is complexed with another protein, and that this complex, which binds thyroxine, migrates in the interalpha area as TBG. When serum is supplemented with purified prealbumin, additional complexing occurs until the receptor protein is saturated. The uncomplexed remainder of the added protein is then free to migrate to its prealbumin position, where it too may bind thyroxine.

Several aspects of these findings seem difficult to explain. First, it has been estimated that the concentration of TBG in normal human serum is 1 to 2 mg. per cent.¹⁶ If so, the concentration of albumin is 2000 times as great. Since tryptophan has been estimated to comprise approximately 0.2 per cent of human serum albumin,⁴² it follows that any aliquot of serum should then contain approximately 4 times as much tryptophan in albumin as in TBG, even if the latter were a pure polypeptide of tryptophan. Unless the tryptophan in albumin is peculiarly refractory to staining, the finding of tryptophan staining only in the TBG zone of normal serum is difficult to understand. Second, little bromphenol blue stain is normally noted in the interalpha area. When serum was supplemented with prealbumin, some staining with both Ehrlich's reagent and bromphenol blue was noted in the prealbumin zone. The intensity of Ehrlich's stain in the interalpha zone was considerably greater than that in the prealbumin position. Nevertheless, no increase in protein staining in the TBG zone was described. Had quantities of protein proportionate to the increased Ehrlich's reaction localized in the TBG zone, at least a moderate bromphenol blue stain would have been expected in this location. It seems possible that prealbumin and TBG, like albumin,⁴³ might bind tryptophan, and that the Ehrlich-positive material that Tata noted might represent bound tryptophan rather than that forming an integral part of the protein molecule.

Our own efforts to confirm Tata's electrophoretic findings have met with little success. When normal serum was electrophoresed in Veronal buffer, the predominant tryptophan stain was localized in the albumin area. Fainter staining was evident in the α_2 - and β -globulin zones. At most, a very faint stain was seen in association with TBG. No differences in Ehrlich's staining were noted when serum was electrophoresed in Tris-maleate buffer, although the localization of added labeled thyroxine was markedly changed.

When serum was enriched with purified prealbumin, Ehrlich's staining appeared in the prealbumin zone. No increase in protein or tryptophan staining was seen in the TBG area. Added labeled thyroxine was abstracted from both TBG and albumin onto TBPA. These findings provided no evidence for an increase in thyroxine-binding, protein, or tryptophan content of interalpha moieties following enrichment of serum with prealbumin.

Tata³³ also studied the immunological characteristics of human prealbumin

and a rabbit antiserum thereto, both prepared by Schultze. With the Ouchterlony agar diffusion technique,⁴⁴ some interaction was found between the antiserum and an interalpha electrophoretic eluate of serum presumed to contain TBG. However, multiple precipitin bands resulted from the interaction of the antiserum and prealbumin. Of these, two appeared to be confluent with the bands developed in the reaction between the antiserum and TBG. Tata interpreted these findings, together with profile counting of labeled thyroxine within the agar plates, to indicate an immunological cross-reaction between prealbumin and TBG. However, the multiple precipitin zones associated with the prealbumin antigen suggest that this preparation was itself immunologically heterogeneous, and was probably contaminated with TBG.

Findings in starch-gel systems. In 1958, Rich and Bearn⁴⁵ reported that, during starch-gel electrophoresis of serum, labeled thyroxine migrated with a prealbumin and with albumin. No binding of thyroxine in the interalpha zone was seen. This finding was interpreted to mean that, in starch gel, TBG migrated to the prealbumin area. Allison³⁴ has recently reported similar findings for human serum and has further indicated that Schultze's prealbumin migrates as the first prealbumin band of human serum. However, Blumberg and Robbins⁴⁶ have reported that, during starch-gel electrophoresis of serum, at least 4 thyroxine-binding zones are seen, of which one is localized ahead and one at the leading edge of albumin. It is evident that these discrepancies between results obtained with the same technique must be resolved before interpretation and correlation with paper electrophoretic findings can be attempted.

Conclusions and summary. Information concerning the interaction of the thyroid hormones with the thyroxine-binding globulin of plasma (TBG), based on findings obtained during paper electrophoresis in Veronal buffer, has been briefly reviewed. The emergence of an additional thyroxine-binding component, the thyroxine-binding prealbumin (TBPA) has been described. The binding properties of TBPA and TBG in normal and abnormal sera as revealed by paper electrophoretic studies in other systems have been described. These findings have been applied to a consideration of current theories concerning the relation between TBG and TBPA.

On the basis of presently available evidence it would appear that there are at least 2 primary thyroxine-binding proteins in normal human plasma: TBG and TBPA. These appear to be separate proteins, differing markedly in their hormonal-binding properties. So rapidly have changes occurred in this field, however, as protein-hormone interactions have been studied by newly developed techniques, that categorical conclusions seem premature. It is to be hoped that future correlative studies with multiple techniques will afford a degree of concordance sufficient to justify conclusive views concerning the transport of the thyroid hormones in plasma.

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ON THE PERIPHERAL METABOLISM OF THYROID HORMONES

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Introduction

The synthesis of radioactive labeled iodothyronines with high specific activity has permitted the administration of physiological doses to experimental animals and the use of such doses has enabled research work on the metabolism of thyroid hormones to progress considerably. The three chief metabolic pathways known for the iodothyronines are dehalogenation, oxidation of the alanine chain, and esterification of the phenolic group.

Deiodination proceeds in all tissues and does not seem specific for a particular halogen atom of the molecule.^{1,2} The presence of iodothyropyruvic and iodothyroacetic acids in some organs and body fluids³⁻⁵ apparently is connected with two processes: (1) oxidative deamination, giving a keto acid, of which (2) the oxidative decarboxylation leads to the corresponding acetic derivative.^{6,7} The presence of these substances in skeletal muscle as well as in liver and kidney, demonstrated by the enzymatic studies of Lardy's group^{8,9} and *in vitro* studies on tissue slices,¹⁰ shows that the oxidative degradation of thyroid hormones is common to all tissues.

The conjugation process can also be considered a general one, since Flock *et al.*^{11,12} detected the glucuronides of triiodothyronine (T₃) and of thyroxine (T₄) in plasma and urine after the administration of these hormones to hepatectomized dogs. However, the formation of glucuronide^{13,14} takes place chiefly in the liver, and the oxidation of the alanine chain occurs chiefly in the kidney; most studies on the metabolism of the thyroid hormones have been devoted to their fate in these organs. The nature of the metabolites formed by the whole tissues is not well defined. Even the fate of the iodothyroacetic acids is not known, except for the fact that they are deiodinated, as are their precursors.^{5,15}

Our purpose here is not to cover all aspects of the metabolism of iodothyronines, but to discuss only certain matters actually being investigated. We shall consider first the initial phase of cellular metabolism: the penetration of iodothyronines into the cells. A survey of the available data on hepatic transformations of the hormones then will be presented in order to define the origin of some iodinated compounds existing in body fluids. Thus, this communication is concerned with two main subjects: (1) the kinetics of penetration of thyroid hormones from the plasma to lymph and to the cells, and (2) the nature of iodinated constituents in the plasma and in the lymph as reflecting partially the hepatic and general metabolism of iodothyronines.

Penetration of 3:5:3'-Triiodo-L-Thyronine and of L-Thyroxine into the Cells

The rate of penetration of thyroid hormones labeled with I¹³¹ into the cells has been studied by following the kinetics of the partition of two iodothyronines in the different body spaces and the kinetics of the elimination of these or of their derivatives in urine and bile.

We injected into thyroidectomized dogs under chloralose anesthesia physiological doses of I^{131} -labeled hormones of high specific activity with a dye (Evans' blue), sodium chloride labeled with Na^{24} , and tritiated water (H^3). The use of these three isotopes and a dye makes it possible to determine in each space the amount of radioiodine, the volume of the different body spaces, and their water content.

The plasma volume (P) has been estimated with sufficient accuracy by the dilution of Evans' blue.¹⁶ The administration of $Na^{24}Cl$, which diffuses freely from plasma into extracellular fluid until an equilibrium is obtained, has allowed the determination^{17,18} of the extracellular space (EC). In the present case, the estimation of radioactivity of Na^{24} in the plasma was complicated by the radioactivity of I^{131} , as both emit β and γ rays, but the proportion of the two radioelements could be estimated from measurements made at two different times, since the half life of I^{131} is 8 days; of Na^{24} , 15 hours. The volume (E) was calculated by subtracting the plasma space P from the space labeled with Na^{24} . At the same time it was possible for us to calculate the I^{131} total plasma radioactivity (R_{plasma}^{131}), knowing the total amount of plasma and the radioactivity in it.

As samples of extracellular fluid are not collected easily, we analyzed the lymph that was in equilibrium with it. The same method of measurement of I^{131} and Na^{24} levels in the lymph allowed the determination of the total extracellular I^{131} (R_{ec}^{131}).

The I^{131} intracellular radioactivity (R_{ic}^{131}) is calculated from the difference between the injected I^{131} (R_{inj}^{131}) and the sum of $R_{plasma}^{131} + R_{ec}^{131} + I^{131}$ excreted by bile and urine (R_{exc}^{131}):

$$R_{ic}^{131} = R_{inj}^{131} - (R_{plasma}^{131} + R_{ec}^{131} + R_{exc}^{131})$$

Injection of tritiated water permits the determination of total body water content (t) by measurement of the radioactivity of H^3 in a sample of plasma when the diffusion equilibrium is reached.¹⁹ The water contents of plasma (p) and of lymph (e) are determined by subtracting the respective dry weights of plasma and of lymph from P and E . The water volume of cells (c) may be expressed as $c = t - (p + e)$.

Circulating thyroid hormones reach the extracellular space at different rates, according to their nature, before they are picked up by the cell receptors. Klitgaard and others have tried to identify metabolites of T_4 in the lymph.²⁰ Gross *et al.*²¹ observed by histoautoradiography that the cellular pickups of T_4 and T_3 are nearly general. The efficiency of the process varies in the different organs; the liver is especially active, as has been shown by several authors.^{11,12}

The previously mentioned techniques have allowed us, with others,^{22,23} to study the kinetics of the partition of T_4 and T_3 between the different water spaces, in physiological doses injected intravenously into thyroidectomized dogs. The experiment, performed on 14 dogs, is as follows. The animals are fasted for 1 day, and then the thoracic lymph duct, bile duct, and urethra are catheterized. Tritiated water is injected. Half an hour later, $Na^{24}Cl$ and the hormone labeled in the 3', 5', or 3' position and in a dose of 0.1 $\mu g./kg.$ are injected. This is followed shortly by injection of Evans' blue. The total body water is determined from a sample of blood taken prior to the hormone

injection. The diffusion of T_4 or of T_3 is checked by determinations made on plasma, lymph, bile, and urine samples taken at different times. Liver biopsy samples are taken for an examination of the penetration of these hormones into liver cells.

FIGURE 1 shows the kinetics of diffusion of T_3 and T_4 in the several spaces during a short time experiment (4 hours).

T_3 disappears rapidly from plasma and, at the same time, appears in the extracellular space. Liver cells concentrate significant amounts of T_3 (at least

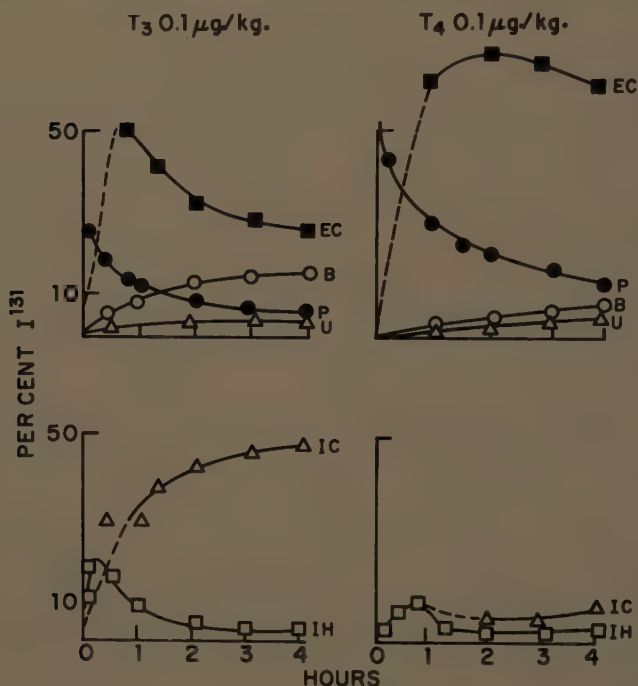


FIGURE 1. Diffusion curves of injected $0.1 \mu\text{g./kg.}$ T_3 and T_4 in the various spaces of thyroidectomized dog. Short time experiment. P = plasmatic space, EC = extracellular space, IC = intracellular space, IH = hepatic intracellular space; B = bile; U = urine.

more than 20 per cent). This concentration is short-lived. The kidneys eliminate very little radioactive material. Intracellular penetration of T_3 is very rapid, approximately 50 per cent in 4 hours.

About 65 per cent of T_4 is present in the extracellular space in the first hour and more than 30 per cent still remains in the plasma space. This phenomenon is quite independent of the fact that T_4 is strongly bound by some plasma proteins. It is not unlikely that the complex thyroxine-binding protein (TBP)- T_4 crosses directly from the capillary wall to the lymph, since we found the same electrophoretic pattern in both fluids. Radioactivity of lymph decreases slowly. Intracellular radioactivity remains low except in liver.

FIGURE 2 shows the results of a long time experiment on other dogs (15 hours). The graphs of this figure illustrate the following results: T_3 or T_4

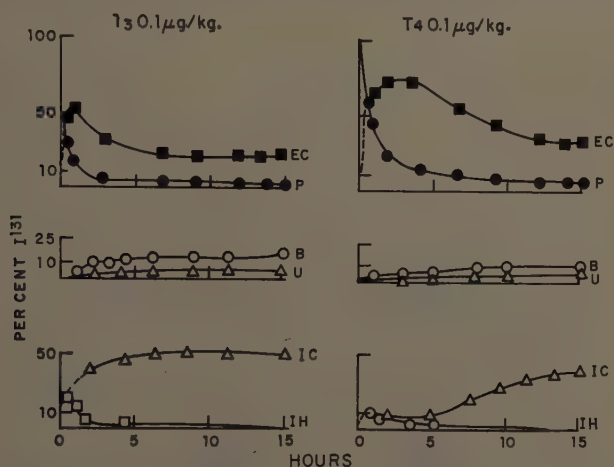


FIGURE 2. Experiment represented in FIGURE 1, through 15 hours. See legend, FIGURE 1, for explanation of symbols.

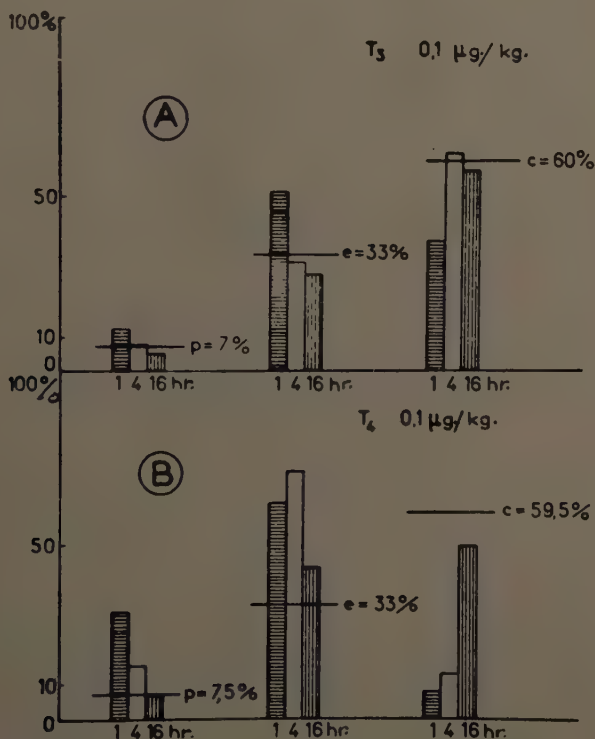


FIGURE 3. Percentage, of total injected T_3 and T_4 labeled with I^{131} , found in the water of different spaces, 1 hour, 4 hours, and 16 hours after treatment. Lines p, e, c show the ratios of water in plasmatic (p), extracellular (e), and intracellular (c) spaces to the total body water, estimated by $H_2^{18}O$.

passes rapidly into the interstitial space. T_3 does not remain there, but is rapidly picked up by the cells. T_4 remains almost completely in the extracellular space for 4 hours. It later begins to penetrate into the cells which at the fifteenth hour, contain only 40 per cent of the radioactivity received.

FIGURE 3 shows the distribution in percentage of the total radioactivity found in the water of different spaces at 1 hour, 4 hours, and 16 hours after injection of T_3 and of T_4 . T_3 is distributed rapidly in the different spaces in

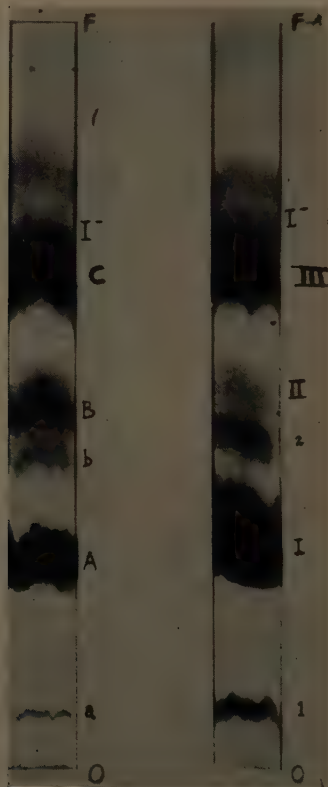


FIGURE 4. Radioautochromatogram of bile samples of rats. *Left*, after injection of T_3 . *Right*, after injection of T_4 . Solvent: collidine/water/ NH_3 . A and I = glucuronides, B and II = pyruvic acids, C and III = mixtures of T_3 or T_4 and esters a, b, 1 and 2 = unknowns.

proportion to their water contents; an equilibrium is reached near the fourth hour and lasts at least until the sixteenth hour.

The partition of T_4 in the plasma and interstitial medium is nearly the same until the fourth hour. Later the radioactivity of I^{131} penetrates into the cells, but the radioiodine concentration by unit volume of cell water is less than this concentration in the plasma and extracellular water.

The rate of intracellular penetration of T_3 is at least 5 times that of T_4 . Thus it may be assumed that the ratio between the times necessary for the entering of T_3 and of T_4 into the cells can explain at least in part the differences between the biological activities of both hormones.

Liver Metabolism of 3:5:3'-Triiodo-L-Thyronine and of L-Thyroxine

The figures given in the foregoing illustrate quantitatively the role liver plays immediately after the injection of T_3 and T_4 . Hepatic cells collect significant amounts of the hormones; these are metabolized in different ways, one of which is of special interest here.

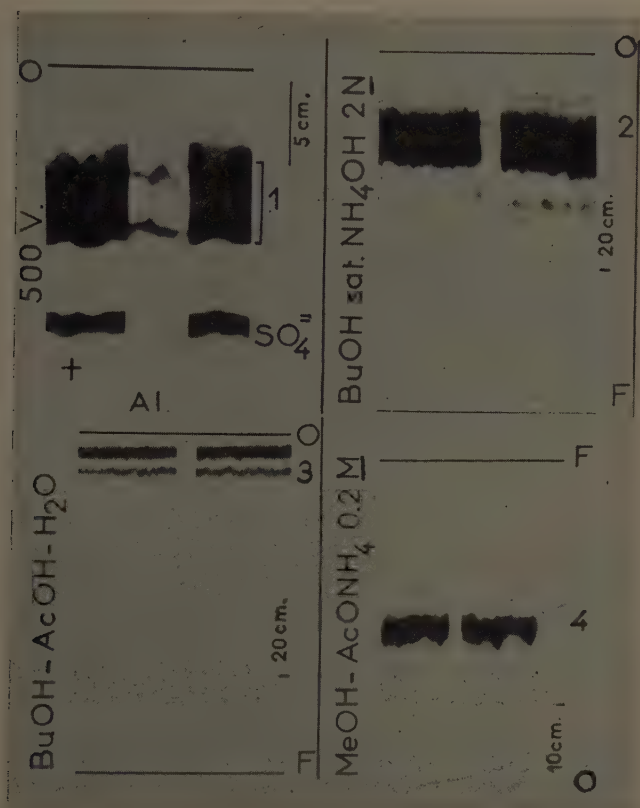


FIGURE 5. Radioautography of bile samples from rats obtained after injection of $Na_2S^{35}O_4$ and 1.3 μg . T_3 . Upper left, preparative electrophoresis in 0.05 M $(NH_4)_2CO_3$ buffer, 5 hours, 500 volts. Upper right, preparative chromatography in *n*-butanol 2 N ammonia after elution of strip 1. Bottom left, preparative chromatography in *n*-butanol acetic acid as solvent of eluate 2. Bottom right, ascending preparative chromatography of eluate 3 in 0.2 M methanol ammonium acetate, pH 6 (1:2, 5). O = origin, F = front.

When thyroidectomized rats are injected with T_3 labeled in position 3' with ^{131}I and their bile is collected by means of a cannula in the bile duct a number of radioactive substances are found. Among these, one has been shown^{3,14} to be a glucuronide; another, the 3:5:3'-triiodothyropyruvic acid.⁴ The most important spot (FIGURE 4) long had been thought the hormone itself, together with iodide. This radioactive zone now has been shown to be the sulfate ester of T_3 —denoted^{24,25} ST_3 —the characterization of which will be described shortly.

Our studies were made possible by the simultaneous injections of T_3 labeled with I^{131} and radioactive sulfate (S^{35}) into thyroidectomized rats. The subsequently collected bile contains the two radioisotopes. The first step in isolating the sulfate was a purification by electrophoresis on paper (FIGURE 5).

In visualizing the radioactivity of the two radioelements, advantage was taken of the different kinds and energies of their radiation. When aluminum foil is placed between the paper and the photographic plate none of the β

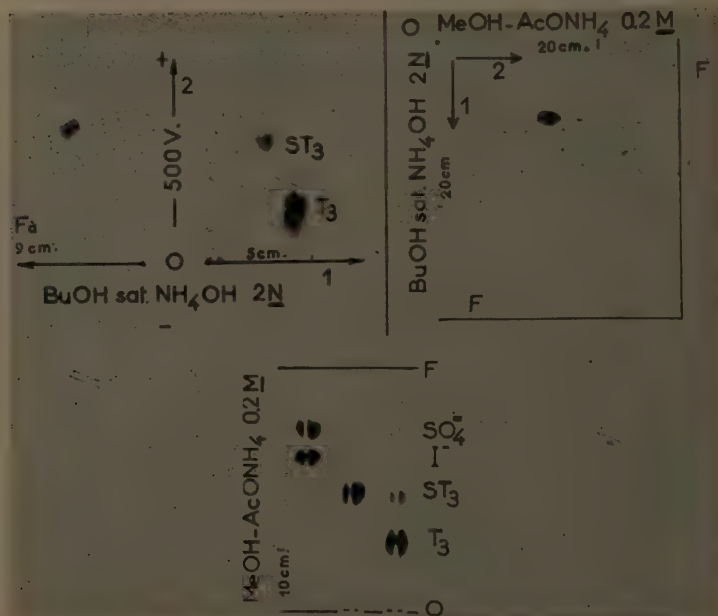


FIGURE 6. Characterization of eluate 4 as ST_3 . Upper left, chromatoelectrophoresis: circular chromatography, with *n*-butanol saturated with 2 *N* ammonia in the first direction, and by electrophoresis in 0.05 *M* $(NH_4)_2CO_3$, pH 9, 500 volts, 5 hours, in the second direction. Upper right, bidimensional chromatography in *n*-butanol saturated with ammonia 2 *N* in the first direction and with 0.2 *M* methanol ammonium acetate in the second. Bottom, ascending chromatography in 0.2 *M* methanol ammonium acetate before (center) and after weak acid hydrolysis (center right) of the eluate 4, standard $S^{35}O_4^-$ and $(I^{131})^-$ (left). Standard ST_3 and T_3 . O = origin, F = front. Spots I^- and SO_4^- are too weak to be seen on the picture.

radiation of S^{35} comes through, while 50 per cent of the β radiation of I^{131} passes, together with all its γ rays.

On the left of FIGURE 5 is the radioautogram of bile after electrophoresis. Strip 1 contains both I^{131} and S^{35} . Under these conditions SO_4^- migrates and iodide goes much farther still. Strip 1 has been eluted and a chromatogram made in *n*-BuOH/ NH_4OH . Several radioactive bands can be seen, a few contain only S^{35} ; this is obvious, since no radioactivity comes through where aluminum foil has been placed. One band contains both S^{35} and I^{131} . An elution of the strip 2 has been performed, and the eluate has been purified by paper chromatography in BuOH/AcOH, where the spot (strip 3) containing both S^{35} and I^{131} has been cut out and eluted. The eluate is chromatographed again in

MeOH/AcONH₄. Here only a single band, numbered 4, remains, containing both S³⁵ and I¹³¹.

This band has been eluted and its chromatographic properties have been compared with synthetic ST₃ in 7 different solvents. FIGURE 6 presents radioautograms of eluate 4. On the right of the circular chromatogram are the spots for synthetic ST₃ and T₃, and on the left, the spot of the eluted strip, which corresponds exactly to the position of the labeled synthetic ST₃. The autochromatogram (*upper right*) represents the spot in two different solvents. The third chromatogram shows, on the left, spots of SO₄⁻ and T₃ (note alu-

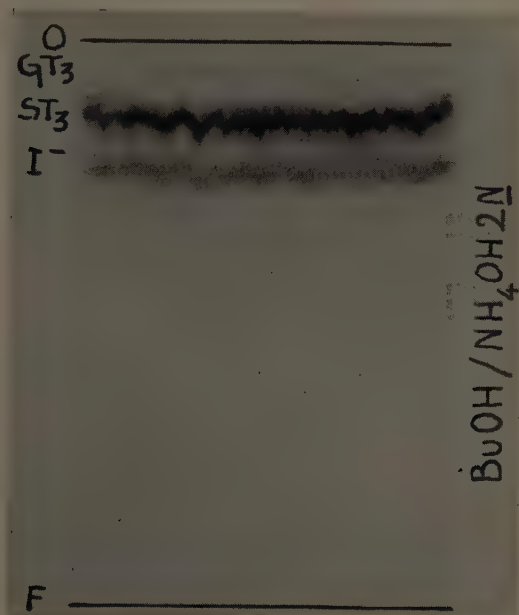


FIGURE 7. Radioautochromatogram of lymph extract 3 hours after injection of T₃ into dogs. Solvent is *n*-butanol saturated with 2 *N* ammonia. O = origin, F = front, GT₃ = position of T₃ glucuronide, ST₃ = position of sulfuric ester of T₃, I⁻ = position of iodide.

minium foil blockage through the middle of the spots). On the center line is the eluted ST₃ band, while on the right line is an hydrolyzate of ST₃ after complete hydrolysis with dilute HCl. Four spots can be noted: one for nonhydrolyzed ST₃, one for the T₃ obtained, one for SO₄⁻, and one for a very little iodide.

In these experiments the thyroidectomized rats received 1 µg. T₃ together with radioactive sulfate. Under such conditions the ratio S³⁵/I¹³¹ is 0.01. To increase this ratio, the low specific activity of S³⁵ in the organism, due to circulating sulfates, was compensated for by large quantities of injected carrier T₃. With a few milligrams T₃ the ratio becomes larger than 1. It should be pointed out that ST₃ is formed not only when pharmacological or emiphiological quantities are administered but even where there can be no doubt that the quantity is physiological, such as 0.01 µg. for a rat. ST₃ may have some importance as a metabolic as well as detoxication product. At

least, it is certainly related to the physiological mechanism of hormonemia regulation; in fact ST_3 may constitute a reserve form of T_3 in the body.

While hepatic esterification of T_4 by sulfuric acid is extremely low, the glucuronide appears in large amounts. Thus, there is an important qualitative difference between the two hormones, a difference that explains some of our findings in plasma and lymph.

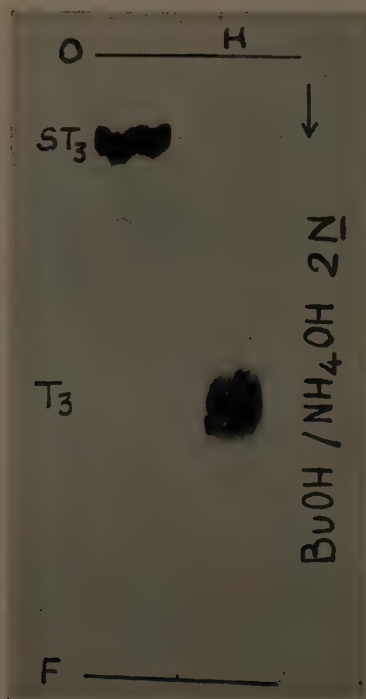


FIGURE 8. Radioautograph of the eluate of strip ST_3 (FIGURE 7) before (left) and after hydrolysis (H) with Taka-Diastase (right), in *n*-butanol saturated with 2 *N* ammonia. O = origin, F = front.

*Plasma and Lymph Iodinated Constituents after Administration of
3:5:3'-Triiodo-L-Thyronine and of L-Thyroxine*

Since radioautographs of dog plasma and lymph taken after administration of T_3 are very similar, we present as examples only those of lymph. We shall later give our quantitative findings on the compositions of the two fluids.

FIGURE 7, concerning lymph constituents at the third hour after injection of T_3 , reveals two main radioactive compounds and a third in much lower amounts. T_3 is absent. The minor product has been identified as the glucuronide of T_3 (GT_3) by its R_f in various solvents and by the products of its hydrolysis by a bacterial β glucuronidase. The chief constituent has the same

R_f as synthetic ST_3 in various solvents and is hydrolyzed by Taka-Diastase with liberation of T_3 , as shown in FIGURE 8.

Autochromatograms of the same type have been obtained from plasma and lymph after administration of T_4 . In plasma collected at the fifth hour we found at least five radioactive substances (FIGURE 9). The lymph collected between the second and the fifth hours has a similar composition in radioactive constituents (FIGURE 10).

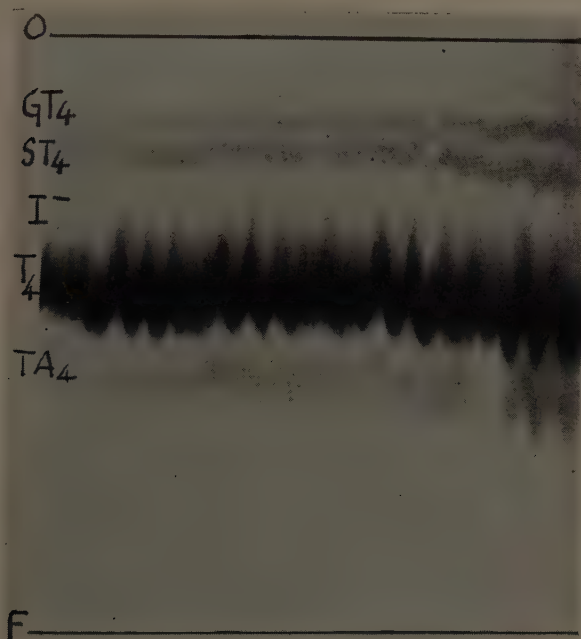


FIGURE 9. Radioautochromatogram of plasma extract 5 hours after injection of T_4 into dogs. Solvent is *n*-butanol saturated with 2 *N* ammonia. O = origin, F = front, GT_4 = position of T_4 glucuronide, ST_4 = position of sulfuric ester of T_4 , I^- = position of iodide, T_4 = position of L-thyroxine, TA_4 = position of tetraiodothyroacetic acid.

The most important spot, D in FIGURE 10, has been characterized as T_4 by chromatography. The spot C has been identified by electrophoresis as iodide. The nature of the other iodinated substances has been specially studied. Spot A has been identified as the glucuronide by chromatography in various solvents, by electrophoresis, and by enzymatic hydrolysis.

Spot B of high electrophoretic mobility (FIGURE 11), regenerating T_4 by Taka-Diastase hydrolysis, is the sulfuric ester of T_4 . It is present only in minute amounts.

The constituent of spot E (FIGURE 12), concentrated after elution, shows on chromatography in BuOH/ NH_4OH the R_f of T_3 , but it migrates differently by electrophoresis. Under conditions where T_3 remains at the origin, the un-

known compound migrates as tetraiodothyroacetic acid (TA_4) and it must be considered to be this derivative and not T_3 .

Two important facts should be pointed out: (1) the plasma or the lymph collected at the fourth or fifth hour after the injection yields untransformed hormone in the case of T_4 , but not in the case of T_3 , and (2) the acetic derivative of T_4 only has been detected. TA_3 is absent.

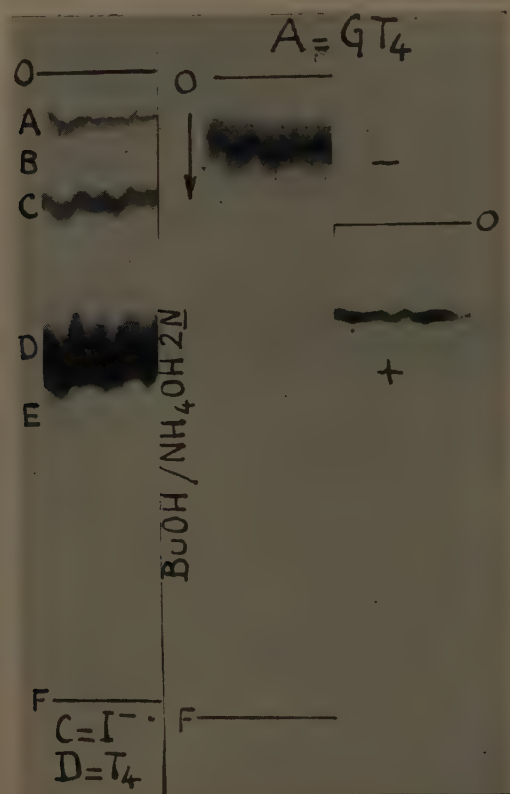


FIGURE 10. I^{131} -labeled products in a sample of lymph taken between second and fifth hours after injection of T_4 into dogs. *Left*, radioautochromatogram of total extract. *Center*, radioautochromatogram of eluate of strip A, thyroxine glucuronide. Solvent is *n*-butanol saturated with 2 *N* ammonia. *Right*, electrophoresis of strip A in 0.05 *M* $(NH_4)_2CO_3$, 5 hours, 500 volts. O = origin, F = front, A, B, C, D, and E = radioactive products.

FIGURE 13 illustrates the disappearance of T_3 from the lymph. Only at the first hour can a significant amount of T_3 be detected, together with I^- , GT_3 , and ST_3 . From the fourth hour T_3 cannot be found. At the eighth hour I^- is the main constituent.

Densitometric determinations on autochromatograms of plasma and lymph collected at different times after injection of T_3 or of T_4 have been made. The results (FIGURE 14) are presented as curves illustrating variations of

the percentage of the iodinated compounds in the two fluids, as a function of time elapsed after the injection and of the total I^{131} injected.

The ratios of the different constituents in both fluids are nearly identical for the same hormone, but the results obtained with T_3 and T_4 are very different. T_3 disappears rapidly at the same time that there is an increase of ST_3 ; in contradistinction, T_4 remains at the high level in the two fluids for a long

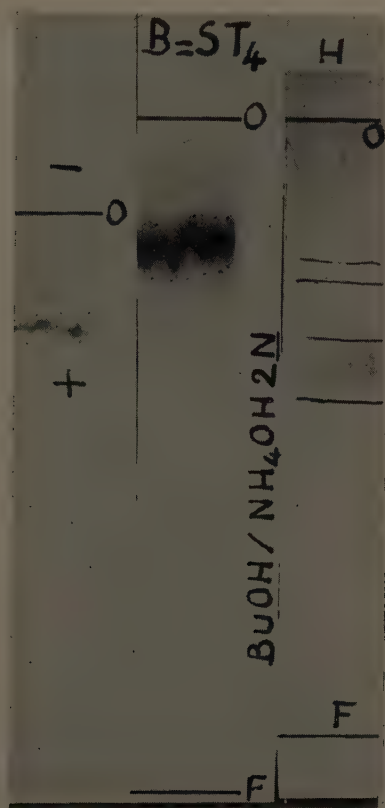


FIGURE 11. Identification of product B (FIGURE 10) as sulfuric ester of T_3 . *Left*, electrophoresis of eluate of strip B, 0.05 M $(NH_4)_2CO_3$, 5 hours, 500 volts. *Center*, radioautogram of strip B. *Right*, radioautogram of strip B after Taka-Diastase hydrolysis (H). Solvent is *n*-butanol saturated with 2 N ammonia. O = origin, F = front.

time (65 per cent in the plasma and 80 per cent in the lymph). TA_4 , ST_4 , and GT_4 are present in minute amounts. Iodide retains 30 per cent of the radioactivity after 9 hours but is formed more slowly from T_4 than from T_3 because of the slower penetration of T_4 into the cells.

Conclusions

The data collected for this paper justify some well-defined conclusions and allow some hypotheses.

The metabolisms of T_3 and T_4 are in many ways dissimilar not only in the liver, but on the general cellular level. T_3 is very rapidly transformed into its sulfuric ester by the liver, and the sulfoconjugate circulating in blood is probably metabolized by cells. T_4 is esterified at a lower rate, as a glucuronide, which participates in an enterohepatic circulation of the hormone.

The penetration of T_3 , and eventually of its ester, into the receptor cells is much faster than that of T_4 . This can explain, at least in part, the greater

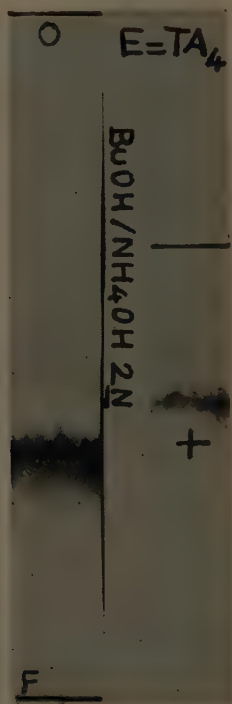


FIGURE 12. Identification of strip E (FIGURE 10) as triiodothyroacetic acid. *Left*, radioautograph of eluate E in *n*-butanol saturated with 2 *N* ammonia as solvent. *Right*, electrophoresis of eluate E in 0.05 *M* $(NH_4)_2CO_3$, 5 hours, 500 volts.

metabolic activity of T_3 . The composition of the lymph, during the first hours after the injection of the hormones, gives a clear picture of this process. Later, the products of cell metabolism return to the lymph, and therefore the picture shows the result of an equilibrium of two streams: fixation of the hormones by the cells and diffusion into the lymph of some degradation products, such as iodide and TA_4 .

With this taken into account, it may be concluded that T_3 is consumed by cells at a much higher rate than is T_4 . ST_3 , and apparently not ST_4 , is part of the hormones transported by blood and very probably is peripherally metabolized.

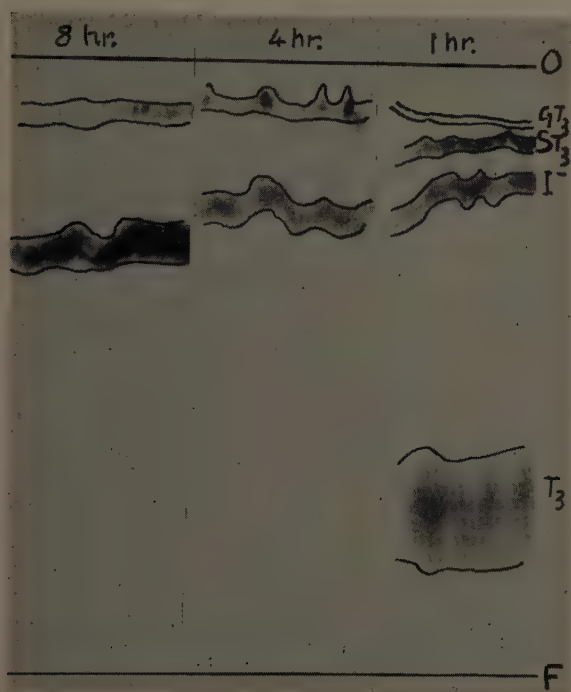


FIGURE 13. Radioautochromatogram of 3 samples of lymph taken at 1 hour, 4 hours, and 8 hours. Solvent is *n*-butanol saturated with 2 *N* ammonia. GT_3 = T_3 glucuronide, ST_3 = sulfuric ester of T_3 , I^- = iodide, O = origin, F = front.

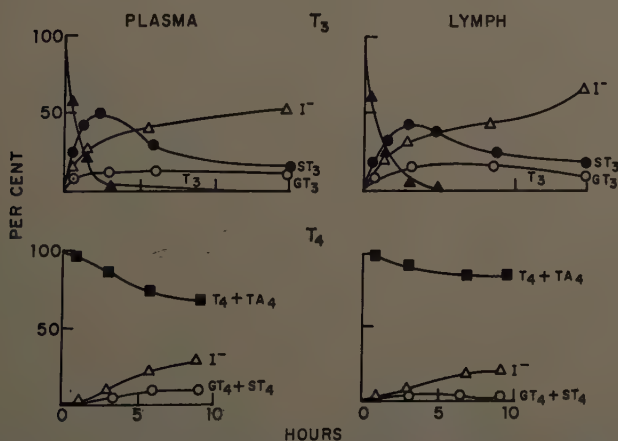


FIGURE 14. Level of different I^{131} -labeled products (ordinate) in lymph and plasma as function of time (abscissa) after injection of T_3 and of T_4 into dogs.

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BIOCHEMICAL APPLICATIONS OF A NEWLY DISCOVERED PROPERTY OF THYROXINE

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Knowledge of the physicochemical properties of thyroid hormones has lagged behind the important advances made recently in the biochemistry and physiology of these substances. I recognized this lack of information on fundamental properties of thyroxine and related substances during my work on enzymic deiodination of thyroid hormones when, at one stage, it became necessary to determine the possibility of a simultaneous, spontaneous deiodination. During such an attempt, a surprising observation was made for which no explanation was readily available. The phenomenon consisted of a virtually instantaneous loss of some of the radioactivity originally present in the thyroxine fraction when I^{131} -labeled thyroxine in an aqueous organic solution was diluted with a relatively large volume of aqueous buffer; furthermore, in a few minutes, without any treatment, most of the lost radioactivity gradually reappeared in the thyroxine fraction, as determined by periodical chromatographic analysis. With some chromatographic solvent systems the I^{131} lost from thyroxine temporarily appeared to be iodide. Because of its importance to the problem of enzymic deiodination, it was decided to seek the cause and mechanism of this hitherto unnoticed transient instability of thyroxine. The object of this paper is not to present any thorough physicochemical study, but to demonstrate that even a relatively superficial knowledge gained of fundamental properties can be applied profitably to considerations of biochemistry and physiology.

Details of the transient instability of thyroxine and related iodophenols, the effect of proteins, and some of the biochemical applications have been described in print recently (Tata, 1959*a*, *b*; Tata and Shellabarger, 1959).

Transient Instability of Thyroxine and Related Iodophenols

This unusual property will be described briefly before the different manners in which it could be applied to other problems are discussed.

The effect is best observed when a small volume of a solution of I^{131} -labeled thyroxine in a mixture of water and 1:2-propane-diol (0.2–0.7 μ moles in 0.03–0.05 ml.) is mixed with about 2.5 to 4.0 ml. of buffered water. The changes occurring in the distribution of radioactivity are followed by paper chromatography of aliquots withdrawn at frequent time intervals after mixing has taken place. The solvent system most commonly used was *n*-butanol-dioxan-ammonia. The result of such a procedure is indicated in FIGURE 1.

The first points of the curves represent the initial loss of radioactivity in the thyroxine fraction followed by the reversal of the process. FIGURE 1 also shows that the rate and extent of both the forward and backward reactions are determined by the *pH* of the aqueous medium. A similar property is exhibited by 3:5:3'-triiodothyronine, and also by 3:5-diiodotyrosine, 3-monoiodotyro-

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sine, and 3:5-diiodo-*p*-cresol. The property of transient instability, however, is most conspicuous with thyroxine. This behavior is to a large extent independent of the concentration of iodophenol, the nature of the buffer, and temperature. On the other hand, it is highly photosensitive, being almost suppressed in the dark. It is not known whether this photosensitivity is in any way related to the decomposition of iodophenol solutions in strong light, first described for thyroxine by Kendall and Osterberg in 1919.

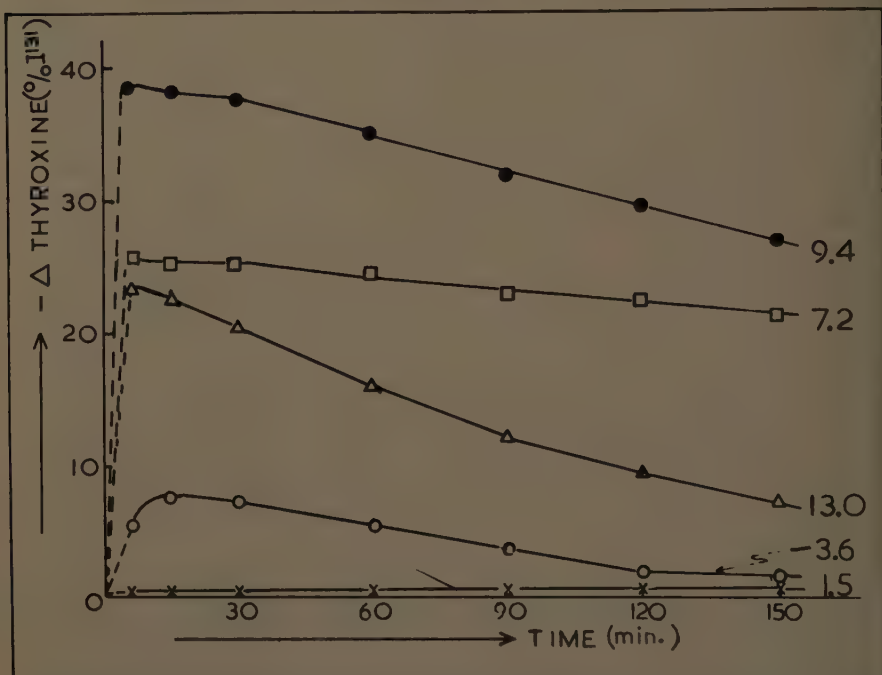


FIGURE 1. Spontaneous disappearance and reincorporation of radioactivity in the thyroxine fraction as a function of time after mixing (at time 0 min.) of I^{131} -labeled thyroxine dissolved in 18 per cent propanediol, with aqueous buffers of different pH values (indicated by figures). The term $-\Delta$ thyroxine expresses the change in the percentage loss of I^{131} in the thyroxine fraction in chromatograms of aliquots withdrawn after the mixing of the radioactive material. Reproduced by permission from *The Biochemical Journal* (Tata, 1959a).

Although the actual mechanism of this unusual reaction is unknown, it is fairly certain that the ionization of the phenolic hydroxyl group of thyroxine and related substances is involved. This conclusion was derived from the following observations:

(1) The pH at which half the maximal rate of initial loss occurred was nearly the same as the pK value for the phenolic-OH group for all the iodophenols studied.

(2) Both the backward or forward reactions could be reversed or produced by a sudden change in pH after the mixing had taken place. The reversal was in the direction predicted by the ionization.

(3) Inclusion of organic solvents that tend to suppress the ionization of the phenolic group had an inhibitory effect.

During these studies it was found that thyroxine and triiodothyronine exhibited more intense absorption in ultraviolet light at the shorter wave lengths of 231 and 227 $m\mu$ than at the generally employed wave lengths of 325 and 318 $m\mu$. The full absorption spectra of the two hormones were not considered in previous studies (Gemmill, 1955; Lardy, 1955), although Adamson *et al.* had pointed out in 1952 the absorption at short wave length for thyroxine. Advantage was taken of the higher extinction at 231 $m\mu$ for thyroxine to demon-

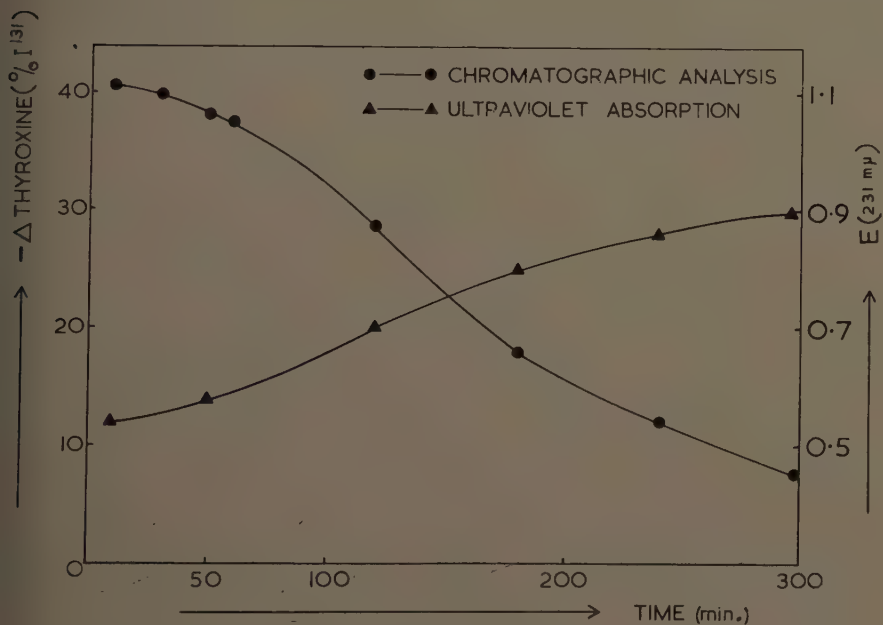


FIGURE 2. Simultaneous chromatographic analysis (○) of radioactivity in thyroxine fraction and the change in ultraviolet absorption (▲) as judged by the extinction at 232 $m\mu$. Reproduced by permission from *The Biochemical Journal* (Tata, 1959a).

strate, by a combination of spectrophotometry and paper chromatography, that the reincorporation of radioactivity in the thyroxine fraction represented a true increase in thyroxine concentration after the initial loss (FIGURE 2).

The nature of the transient product formed remains unknown. Although it had chromatographic properties (in *n*-butanol-dioxan-ammonia) similar to those of inorganic iodide, the following results rule out the possibility of a spontaneous and self-reversible deiodination or exchange of radioactivity: (1) the product had an electrophoretic mobility different from that of iodide; (2) if the 5 to 10 per cent of contaminant radioiodide were accounted for, no I^{131} could be extracted in CS_2 after oxidation with Fe^{3+} ions or H_2O_2 ; (3) reducing agents had no effect on the forward or backward reactions; (4) the constant fraction of I^{131} appearing as the transient product in the presence of varying

amounts of stable iodide excluded the possibility of a reversible exchange of radioactivity. Identification of the transient product would help greatly in elucidating the nature of this phenomenon.

Transient Instability of Thyroxine and Interaction with Proteins

The above observations were followed by attempts to determine whether the transient instability could occur in the presence of biological media. In the first experiments, different amounts of human serum were incorporated in the aqueous buffer to which thyroxine was added. The results showed that human serum masked the effect of the ionization of the phenolic group of thyroxine by inhibiting the initial loss or accelerating the re-formation, as illustrated in FIGURE 3. If serum was present in the aqueous buffer before radioactive thyroxine was added (FIGURE 3A), the disappearance of I^{31} was inhibited; on the other hand, if it was added to the buffer after the mixing (FIGURE 3B), it caused the immediate reappearance of all the I^{31} in the thyroxine fraction. The extent of the inhibition of forward or the acceleration of backward reactions was related directly to the amount of human serum added. This effect was observed above pH 6.0, but was absent below about pH 5.0, probably indicating the isoelectric zone of the proteins of human serum that may be responsible for the inhibition. The inhibition of initial loss of radioactivity by any agent added has been termed "stabilization" of thyroxine and is also applicable to 3:5:3'-triiodothyronine, although the transient instability is less marked for the latter compound.

At this stage it was decided to determine whether the "stabilization" effect of human serum was related to the well-known property of thyroxine-binding by certain serum proteins (Robbins and Rall, 1957; Pitt-Rivers and Tata, 1959). For this purpose the stabilizing action of isolated human serum proteins was examined simultaneously with their thyroxine-binding activity by the classic methods of paper electrophoresis or protein precipitation. As shown in FIGURE 4, the stabilization of thyroxine produced by isolated protein fractions does indeed reflect their thyroxine-binding property. The α -globulin-rich Cohn Fraction IV-6 (and also Fractions IV-4 and IV-9) and human serum albumin had been previously shown by paper electrophoresis to be the major thyroxine-binding protein components (Freinkel *et al.*, 1955). The high thyroxine-binding potency of Schultze's prealbumin used in this work was confirmed in other studies (Tata, 1959c), although it is not certain whether it represents the same prealbumin fraction observed to bind thyroxine in starch-gel electrophoresis (Rich and Bearn, 1958). At constant pH and concentration of thyroxine, the extent to which thyroxine is stabilized is a function of protein concentration (FIGURE 5). Measurement of protein-bound or free thyroxine by electrophoresis or protein precipitation, when increasing amounts of protein were added,

FIGURE 3. (A) Inhibitory effect of the presence of human serum on the spontaneous disappearance of a part of the radioactivity in the thyroxine fraction. \circ : Buffer (tris maleate pH 7.5) plus human serum (diluted 1:4); \square : buffer alone. Reproduced by permission from *The Biochemical Journal* (Tata, 1959b). (B) Accelerated re-formation of radioactive thyroxine in chromatograms as a result of the addition of human serum after the initial disappearance of thyroxine had occurred. At the point indicated by the left arrow, sufficient human serum was added to obtain a final fourfold dilution; at the point of the right arrow, in another reaction vessel, the added human serum gave a final 1:10 dilution. The dashed line represents the slow reappearance of thyroxine radioactivity in the serum-free control. Reproduced by permission from *The Biochemical Journal* (Tata, 1959b).

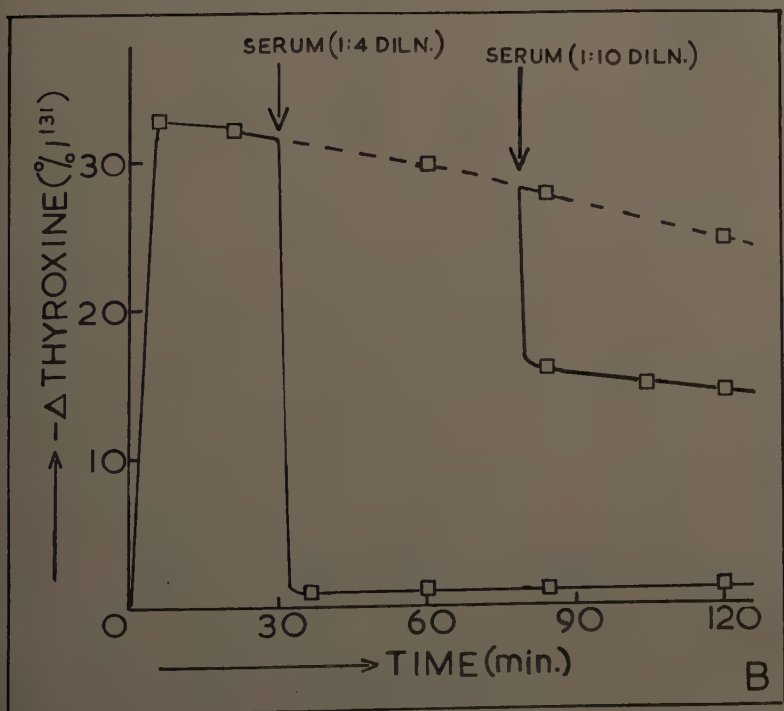
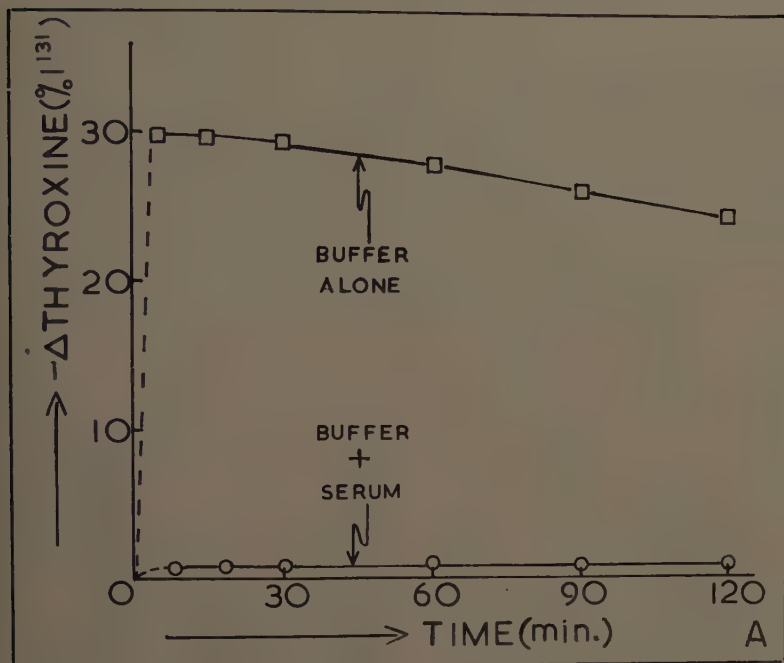


FIGURE 3.

proved that the fraction of thyroxine stabilized was the same as that bound to the protein. This is valid for paper electrophoresis only at relatively high concentrations of protein since, at low concentrations, the electrophoretic method tends to give lower values for protein-bound thyroxine, principally owing to the relatively increased adsorption of thyroxine by paper. The curves seen in FIGURE 5 were obtained for very small amounts of thyroxine ($6-9 \times 10^{-9}$ M); if the same experiments were performed by mixing increasing amounts of thyroxine, there was a shift toward the right of the intercepts on the abscis-

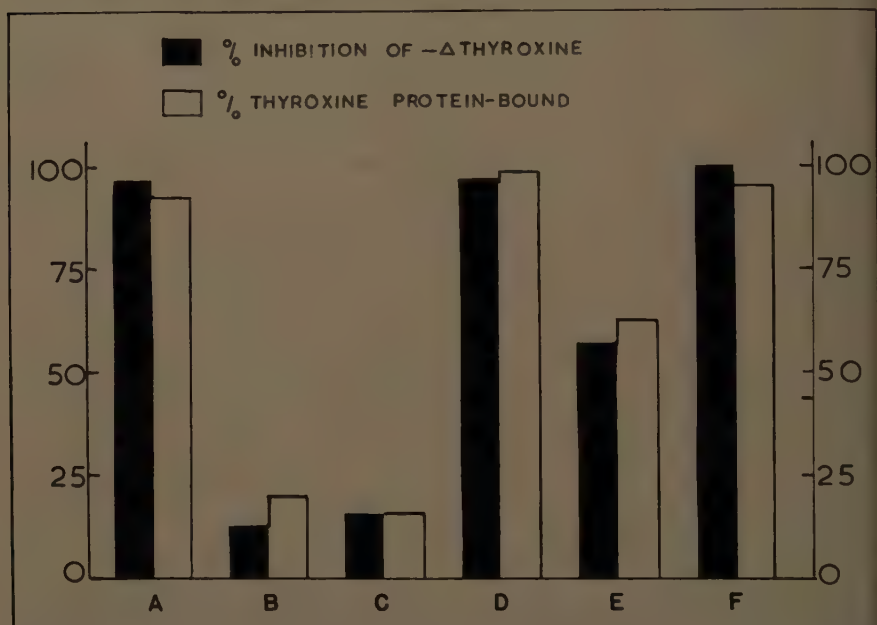


FIGURE 4. Relationship between the stabilization effect (black bars) and thyroxine-binding (open bars) by human serum and some of its protein fractions. Stabilization was measured as the percentage inhibition of the initial loss of radioactivity, 5 min. after mixing, in the thyroxine fraction, as judged by paper chromatography. The percentage of thyroxine protein-bound was determined by paper electrophoresis of the same samples. (A) Whole human serum (28.0 mg./ml.); (B) γ -globulin (5 mg./ml.); (C) β -globulin (5 mg./ml.); (D) Cohn Fraction IV-6 (5 mg./ml.); (E) human serum albumin (15 mg./ml.); (F) prealbumin (2.5 mg./ml.). Reproduced by permission from *The Biochemical Journal* (Tata, 1959b).

sae, but the slope of the curve did not vary much. The extent of shift of intercepts varied from one protein fraction to another.

The pH-dependency of stabilization is different for different protein fractions. For whole serum and Cohn Fraction IV-6 there is no stabilization at pH 5.0, maximum stabilization occurs at pH 9.0, and it is pH 5.0 and 11.5 for albumin, respectively. An interesting effect was observed with γ -globulin. This protein, which normally does not stabilize or bind thyroxine up to a pH of about 9.5, rapidly begins to acquire strong stabilization properties when the pH is raised to 11 or 12. This was confirmed by paper electrophoresis at high pH, and the effect underlines a possible "opening up" of binding groups in the protein.

Biochemical Applications

The biochemical applications reported below relate to the problems in which I was particularly interested at the time of these studies. The same principles could be applied, however, to other problems.

Binding of thyroid hormones by proteins. The study in recent years of binding of thyroid hormones to serum proteins and their transport has been largely

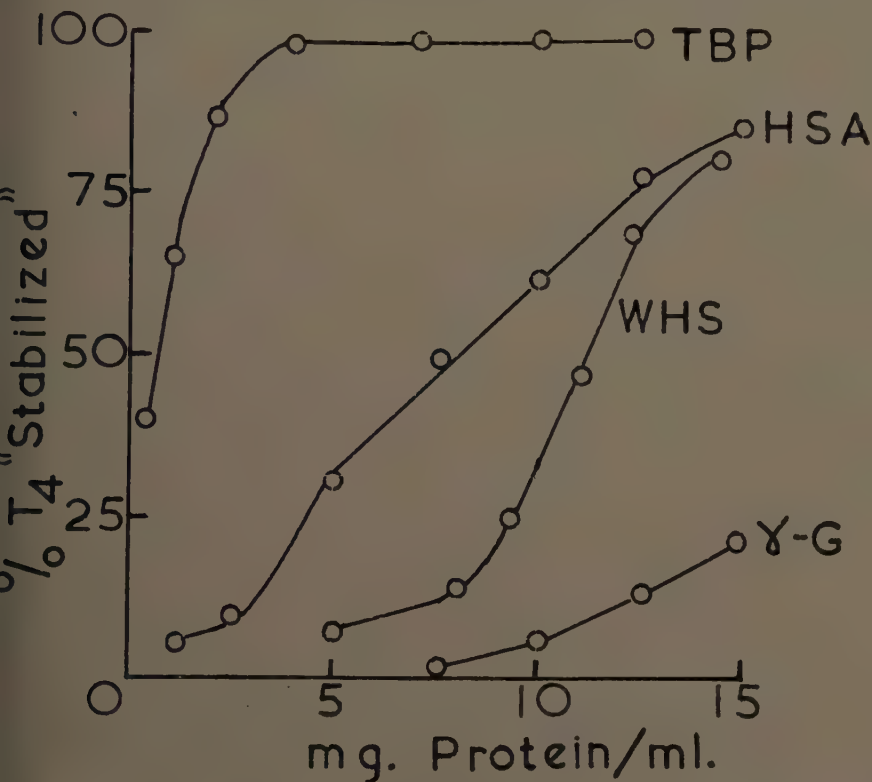


FIGURE 5. Effect of variation of protein concentration on stabilization of thyroxine. In general, the slope indicates the intensity of interaction between the protein sample and thyroxine. WHS: whole human serum; TBP: an extract of Cohn Fraction IV-9 with high thyroxine-binding power; HSA: human serum albumin; γ -G: γ -globulin.

due to the use of the paper electrophoretic technique, partly because of its merits and partly because of the lack of any satisfactory alternative method. Paper electrophoresis as applied to thyroid hormone binding to proteins (Robbins and Rall, 1957; Tata, 1960) has certain inherent defects that can be overcome by the use of the method of thyroxine stabilization, the advantages of which are listed below.

(1) The method of stabilization offers a simple means of comparing accurately the thyroxine-binding powers of whole serum or isolated proteins. The principle is illustrated in FIGURE 5. The slope obtained by measuring stabilization at increasing protein concentration is a measure of the affinity of the protein

or the medium for thyroxine. Identical slopes indicate that the same component is involved as for the thyroxine-binding protein (TBP)-rich extract and whole serum; the reverse is reflected in the less steep slope obtained for albumin (FIGURE 5).

As a first example, FIGURE 6 shows the comparison of thyroxine-binding powers of normal and pregnancy sera. The curves obtained are almost parallel

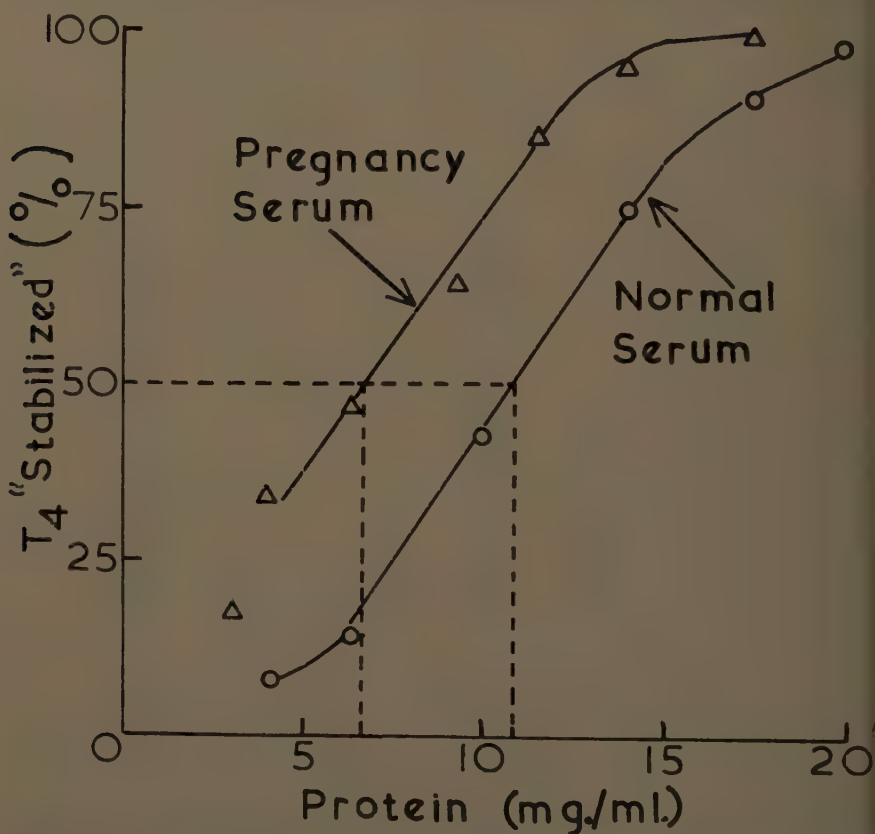


FIGURE 6. Comparison between thyroxine-binding powers of serum from a normal subject and that from a pregnant subject (29 weeks), as measured by the stabilization of thyroxine in the presence of increasing amounts of the sera.

thus indicating that the principal thyroxine-binding component is identical in both serum samples. The shift to the left, of the curve obtained for pregnancy serum, indicates that there is more of this component. Only half as much pregnancy serum as normal serum is needed to achieve a 50 per cent stabilization of thyroxine. The roughly twofold increase in serum thyroxine-binding power observed during pregnancy is in agreement with results previously obtained (Dowling *et al.*, 1956; Robbins and Rall, 1957).

The second example is the case where the slopes are not identical. In FIGURE 7 is shown the comparison of thyroxine-binding powers of human and chicken

sera. The slopes of the two curves are not parallel, which indicates that thyroxine is bound in the two sera to components of different nature. Also, the less-steep slope of the chicken serum curve shows that the hormone is bound less firmly in chicken serum than in human serum. In fact, it was shown by Tata and Shellabarger (1959) that avian serum is found to lack a thyroxine-binding protein of the type found in mammalian serum and that albumin is

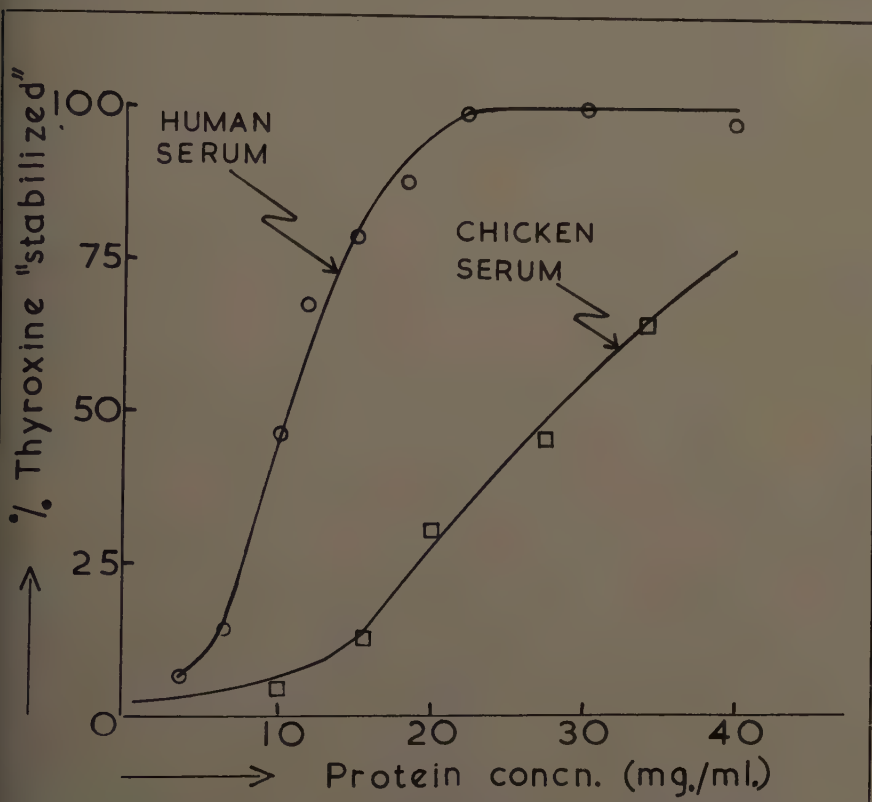


FIGURE 7. Thyroxine-binding power of human and chicken sera at different dilutions expressed in terms of percentage stabilization of thyroxine. \circ : human serum; \square : chicken serum. Reproduced by permission from *The Biochemical Journal* (Tata and Shellabarger, 1959).

the principal circulating thyroxine-binding protein in birds. These facts form the basis of an explanation to account for the differences in response between mammals and birds as regards the physiological potencies of thyroid hormones.

(2) The stabilization method of thyroxine-binding to proteins permits a better distinction between binding "affinity" and "capacity." With paper electrophoresis, the binding capacity or relative affinities of serum proteins have been measured by adding increasing amounts of thyroxine to displace a small amount of radioactive thyroxine from TBP or any other protein (Albright *et al.*, 1955; Robbins and Rall, 1955; Freinkel *et al.*, 1955). The disadvantage of such a

procedure is that the true affinity of a protein for thyroxine can be masked by its binding "capacity" or the saturation level, especially if the binding protein with a high affinity can be saturated easily, as is the case with TBP. This difficulty can be obviated by measurements of thyroxine stabilization, as in FIGURES 5, 6, and 7, at a constant and very low concentration of thyroxine. In all these examples, the slopes to a large extent are a measure of the affinity. The effect of increasing thyroxine concentration would be to shift the position of the whole curve without a significant change in slope.

(3) Cellulose, especially in alkaline media, exhibits a strong affinity for thyroxine and related substances. The resulting interference in paper electrophoresis is negligible when large amounts of protein (as in human serum) are separated, but the "competition" effect (resulting in trailing) is intense when only a small amount of protein is available. Thus electrophoresis of extravascular fluids of low protein content such as cerebrospinal fluid (CSF) necessitates their concentration (often as much as one hundredfold) and thus entails the risk of denaturation. With the method of stabilization it was possible to compare the thyroxine-binding power of CSF with that of serum after only a fivefold concentration (Tata, unpublished). About 2 to 3 times less CSF than serum protein was necessary to bring about the same amount of stabilization caused by human serum, a result compatible with previous studies on thyroxine-binding in human CSF (Alpers and Rall, 1955; Robbins and Rall, 1957). For the same reason, the stabilization method is more suitable for a comparison of thyroxine-binding power of isolated proteins available in only small amounts, such as certain prealbumin fractions or the TBP-rich extract shown in FIGURE 5.

(4) As mentioned earlier, the intensity of binding or stabilization by proteins is influenced by the H^+ -ion concentration of the medium. Most paper electrophoretic separations have been performed in alkaline media around pH 8.6. At this pH the binding intensity is somewhat higher than at pH 7.4; for albumin it is enhanced by 25 to 30 per cent. Measurements by the stabilization method, as illustrated in FIGURES 5 to 7, were made at physiological pH and could also be made in a physiological saline medium, although the influence of ionic composition of the medium on thyroxine-binding by proteins is not yet known. Since temperature does not significantly affect the transient instability of thyroxine, all the above measurements have been made by the new method at $37^\circ C$.

The advantages cited above are qualified by the following consideration. The method of stabilization gives a measurement of the over-all binding of thyroxine in a mixture of proteins, but does not distinguish the contributions of individual components. For this reason, it could be profitably used in some cases only in combination with electrophoretic or chemical methods of separation of proteins.

Thyroxine-binding by nonprotein substances. By means of the new method it is possible to study the binding of thyroxine to nonproteins. The example of the plasma substitute, polyvinylpyrrolidone (PVP) is quite illustrative. The avidity of this polymer for thyroxine was first observed in experiments in which it was used to concentrate protein solutions containing the hormone by dialysis against PVP. As shown in FIGURE 8a, PVP exhibits an intense degree

of inhibition of instability of thyroxine, quite similar to that of whole serum and thyroxine-binding proteins. On the basis of the stabilization curves, it could be predicted that PVP, if added in sufficient amount to human serum, would compete with the thyroxine-binding protein (TBP) for the available thyroxine. Such is indeed the case (FIGURES 8*b* and *c*). When 7.5 mg. of

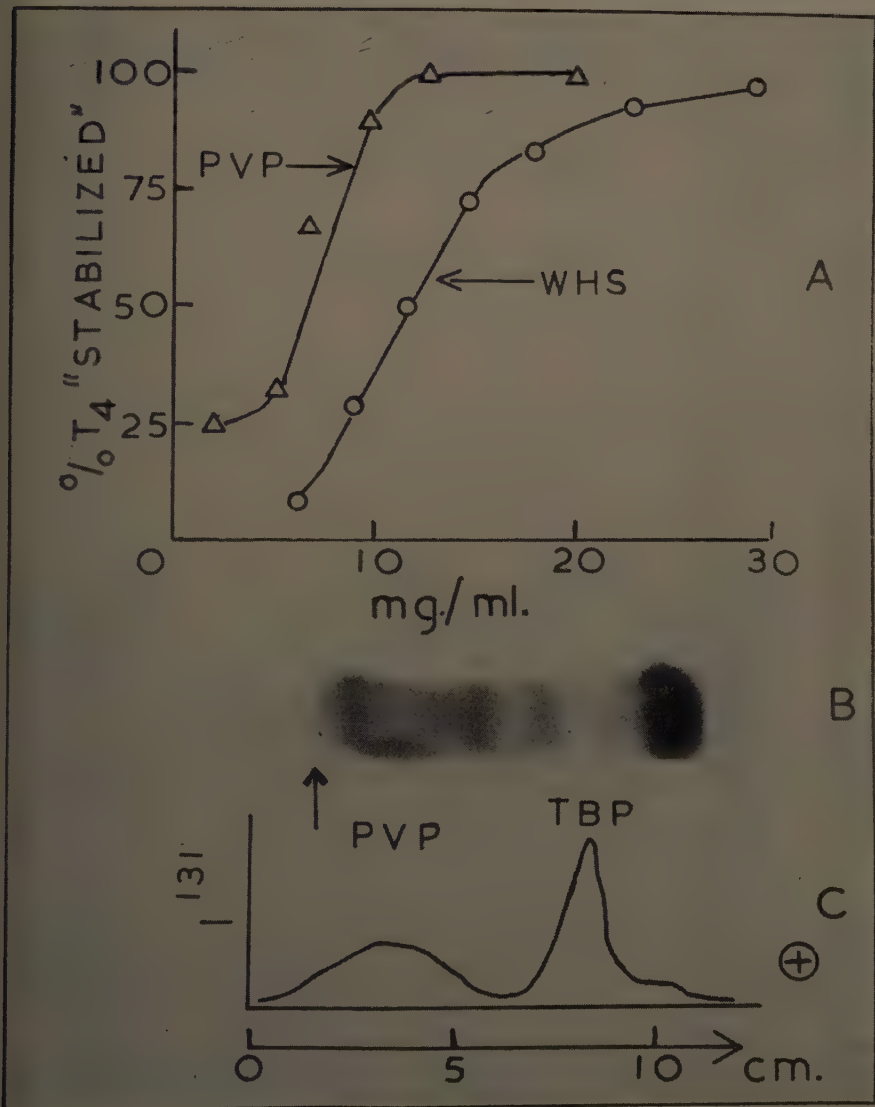


FIGURE 8. (A) Stabilization of thyroxine by polyvinylpyrrolidone (PVP) and whole human serum (WHS) at different concentrations; (B) distribution of serum proteins on paper electrophoresis of a sample of twofold diluted human serum to which PVP was added in the concentration of 7.5 mg./ml.; (C) distribution of thyroxine radioactivity in the paper strip in the zones of PVP and serum TBP (thyroxine-binding protein).

PVP was added to 1 ml. of a 50 per cent dilution of human serum in 0.15 *M* NaCl containing a trace amount of radiothyroxine, about 45 per cent of the radioactivity which would normally be all in the TBP zone was found to be displaced in a different and broad zone on paper electrophoresis. This zone

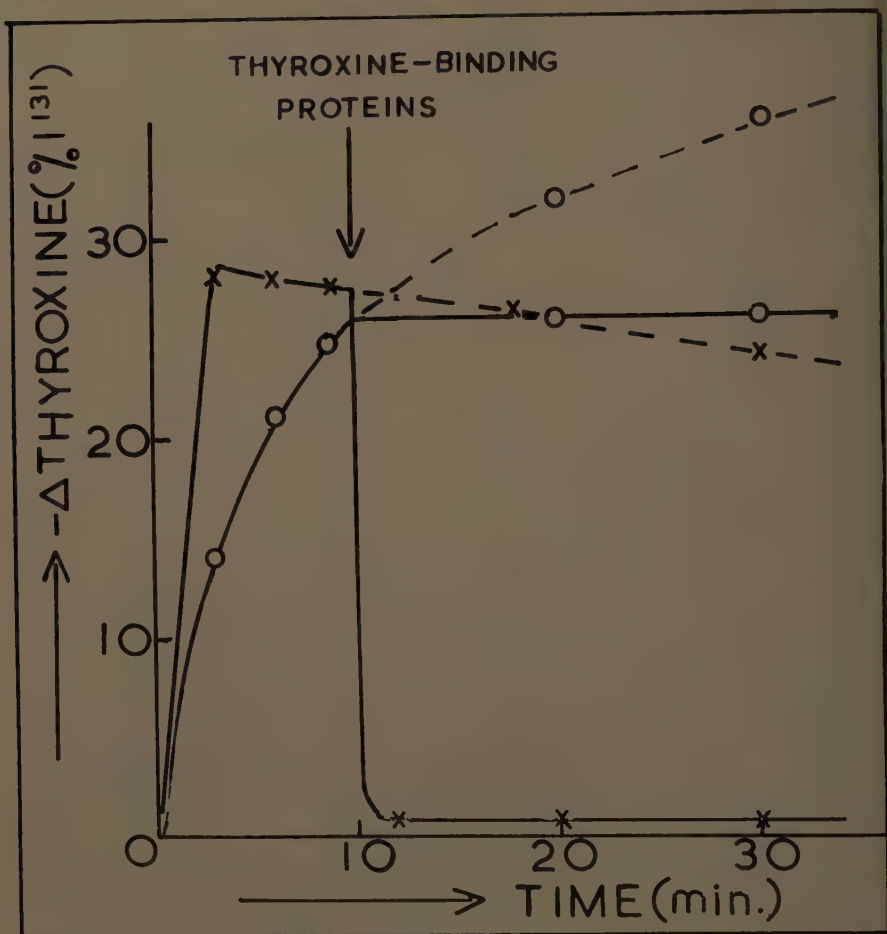


FIGURE 9. Distinction between true enzymic deiodination (O—O, O---O) and transient loss and re-formation (X—X, X---X) of thyroxine by addition of thyroxine-binding proteins, 10 min. (vertical arrow) after either reaction had started. The broken lines show how the two reactions would proceed if no protein were introduced. The same amount of radioactive thyroxine was added in each case. Reproduced by permission from *The Biochemical Journal* (Tata, 1959b).

was found to correspond to the position of PVP on the paper electrophoregram as visualized by its absorption in ultraviolet light or the brown coloration with phthalic anhydride.

Distinction between enzymic deiodination and transient instability of thyroxine. Consideration thus far has been restricted to the property of thyroxine-binding proteins and other substances of preventing the transient instability of thy-

roxine if they are present in the aqueous buffer before thyroxine is added (FIGURE 3a). The second property of these substances to accelerate the reformation of thyroxine (FIGURE 3b), once the change has taken place, can be used to distinguish between true enzymic deiodination and the self-reversible loss of thyroxine, as studied by paper chromatography.

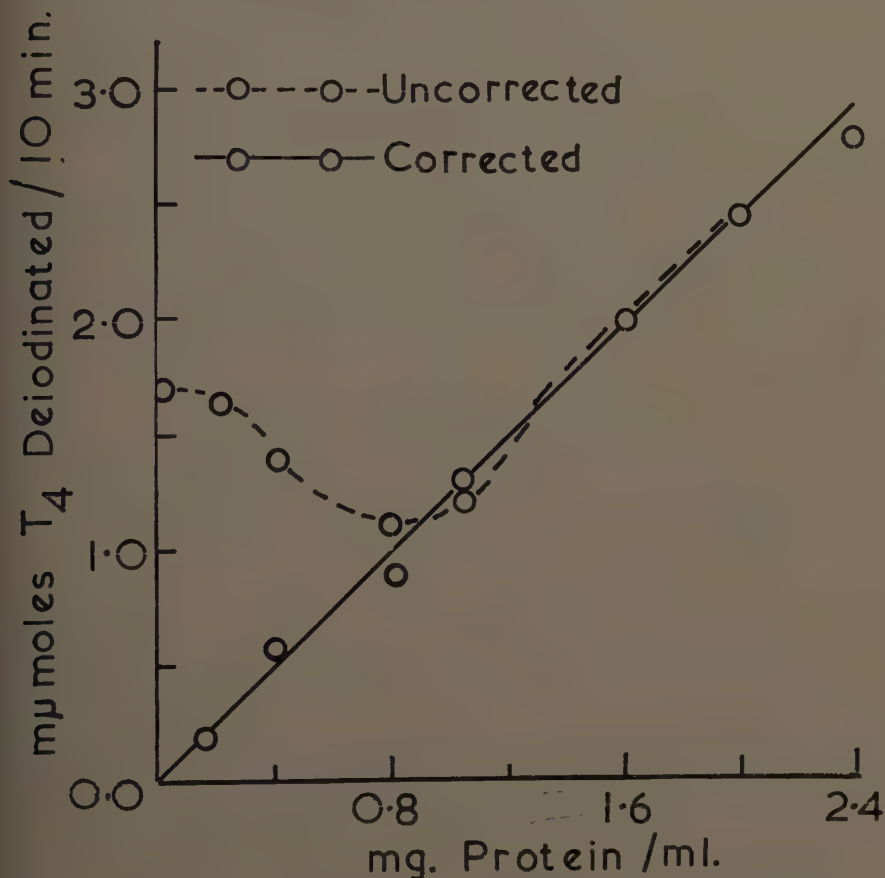


FIGURE 10. Application of the principle described in FIGURE 9. The uncorrected values represent the apparent amount of thyroxine deiodinated after incubation with increasing amounts of a crude preparation of rabbit skeletal muscle deiodinase. For the curve obtained after correction, a small amount of human serum (sufficient to bind all thyroxine) was added to each sample 1 min. before the aliquots were withdrawn for paper chromatographic analysis.

The principle of this application is illustrated in FIGURE 9. The enzyme preparation used was the Fe^{2+} - and flavin-dependent thyroxine deiodinase prepared from rabbit skeletal muscle (Tata, 1959d). In both true and apparent deiodination, most of the radioactivity of thyroxine lost appears as "iodide" on paper chromatograms. If whole serum, or any thyroxine-binding protein fraction from it, is added some time after thyroxine has been incubated with the enzyme, the reaction of deiodination is "frozen." No change occurs there-

after in the amount of radioactivity in the thyroxine fraction; the fraction of I^{131} in iodide or other compounds also does not vary. On the other hand, the original amount of I^{131} is restored in the thyroxine fraction, with a concomitant disappearance in the iodidelike fraction, if the reaction represents transient instability. It was shown by paper electrophoretic analysis that the thyroxine remaining in the case of enzymic deiodination was bound to the thyroxine-binding protein added; besides being "stabilized," protein-bound thyroxine is unavailable to the action of deiodinase.

If the amount of deiodinating enzyme is relatively large, as in FIGURE 9, the extent of both reactions occurring simultaneously is negligible. However, the reactions can be superimposed when small amounts of enzyme preparation are used. The two processes have been distinguished by a test based on the principle described above and illustrated in FIGURE 10. It concerns the relationship between concentration of deiodinase and initial rate of deiodination. It will be observed that a rather unusual U-shaped form of curve was obtained without any correction for transient instability accompanying enzymic deiodination at low levels of deiodinase, but if a small amount of serum or a TBP fraction is added at the end of the incubation, the corrected values will then give a linear relationship. Such a procedure has now been applied routinely to all studies of the purification and properties of thyroxine deiodinase, thus eliminating possible confusion from simultaneous occurrence of enzymic and spontaneous reactions. Hence it is fitting to end this review with an application to the problem of enzymic deiodination, since it is the study of this problem that was responsible for the discovery of the unusual property of thyroxine and related iodophenols.

Conclusion

A hitherto undiscovered property of thyroxine and related iodophenols has been described. It consists of a self-reversible loss and re-formation of the iodophenol when a small volume of its solution in an organic solvent is mixed with a large volume of aqueous buffer. Ionization of the phenolic hydroxyl group is involved in the transient instability which is inhibited by proteins and other substances that bind thyroxine. Although the cause and mechanism of the phenomenon remain unknown, it has been successfully applied to the elucidation of problems of interaction between thyroid hormones and proteins and enzymic deiodination of these hormones.

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Part III. Cellular Mechanisms in Actions of Thyroid Hormones

THYROXINE AND THE SWELLING AND CONTRACTION CYCLE IN MITOCHONDRIA*

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Our current interest in the swelling and contraction cycle of mitochondria and the effect of thyroxine resulted from earlier studies on the uncoupling action of thyroxine on oxidative phosphorylation. In 1955 an observation was made that put the mechanism of action of thyroxine on mitochondria in a new perspective.¹ In brief, it was found that oxidative phosphorylation accompanying electron transport from β -hydroxybutyrate to oxygen could be observed to take place in a submitochondrial system, namely, in fragments prepared from liver mitochondria by the action of digitonin. In striking contrast to its uncoupling action on intact mitochondria, thyroxine was found to have absolutely no uncoupling effect on phosphorylation as it occurred in the fragments, although dinitrophenol was fully active.² Since the fragments are both morphologically and enzymatically simpler than the parent mitochondria, it appeared that the uncoupling action of thyroxine is not a direct one on some intermediate or enzyme concerned in oxidative phosphorylation, but rather is an indirect effect. A similar case came to light: Ca^{++} , even in very high concentrations, failed to uncouple phosphorylation in the digitonin fragments, but is well known to uncouple in intact mitochondria. McMurray *et al.* later confirmed the failure of thyroxine to uncouple phosphorylation in mitochondrial subfragments prepared by sonication.³

A variety of explanations could be invoked for this differential effect of thyroxine. For example, possibly an enzymic transformation of thyroxine into the true uncoupling agent takes place in intact mitochondria and cannot occur in the submitochondrial fragment for lack of the necessary enzyme.⁵ In any case, a possible basis for an explanation was soon found when it was observed that thyroxine in very low concentrations could cause rapid swelling of isolated rat liver or kidney mitochondria suspended in isotonic sucrose buffered at pH 7.4.^{1,4} Very significantly, it was found also that dinitrophenol (DNP) did not cause swelling of mitochondria under the same conditions; in fact, DNP was a protective agent and prevented swelling induced by thyroxine. These experiments showed clearly that these two agents, which can uncouple phosphorylation in intact mitochondria, differ not only in their action on submitochondrial fragments but also in their action on mitochondrial structure and permeability. It was therefore postulated that thyroxine is not simply an analogue of DNP in its action, but has a separate and distinct (and possibly opposite) mode of action that is exerted primarily on some property of the mitochondrial mem-

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brane structure that leads to swelling and that can also lead to uncoupling, but in an indirect or secondary manner.^{1,5,6}

A great deal of new information on mitochondrial swelling and contraction has been accumulated in the brief period of the last 2 years. This newer information is summarized in the following, with special reference to the action of thyroxine. In essence, recent developments point to the view that the morphologic configuration and enzymatic activity of mitochondria are the result of a dynamic balance between enzymatically geared processes of swelling and contraction.

Mitochondrial swelling. Mitochondria isolated from a variety of animal tissues by the sucrose method swell when placed in media of isotonic sucrose or KCl buffered near neutrality. The mitochondria of liver and kidney can swell rapidly to larger volumes, up to four- or fivefold and, as will be pointed out later, can be enzymatically contracted again. The mitochondria of heart and skeletal muscle also swell and contract, but the amplitude of this process is very much smaller than in liver or kidney mitochondria. Brain mitochondria show only a very small amplitude of swelling *in vitro*.⁷

Swelling of liver mitochondria occurs in two major phases with quite different characteristics. The first and rapid phase is the prompt adjustment (swelling or shrinking) of the mitochondrial volume to the osmotic pressure of the medium and to the nature of the external solutes, whether permeant or impermeant. This adjustment is complete in a matter of seconds or minutes. The liver mitochondrion behaves like an ideal osmometer if a rather large osmotic "dead space" of about 40 per cent is assumed.⁸⁻¹⁰

The second phase of mitochondrial swelling is much slower, nonosmotic, and enzymatically controlled, specifically by the action or condition of the respiratory chain responsible for electron transport and coupled adenosine triphosphate (ATP) production; these chains are present in the mitochondrial membrane. It is the phase ordinarily referred to when the word swelling is used;¹¹ purely osmotic changes are often superimposed. This phase of mitochondrial swelling is discussed in further detail in the following.

Swelling of rat liver mitochondria suspended in buffered isotonic sucrose is induced by the addition of one of a few substances of physiological origin, and it is of interest that these substances are effective swelling agents *in vitro* in concentrations in which they exist intracellularly. These substances are inorganic phosphate,^{12,13} Ca^{++} ,¹² reduced glutathione,¹⁴ and thyroxine.^{1,11} Of these, thyroxine is by far the most potent, since detectable swelling effects may be observed at concentrations as low as $10^{-8} M$, or less than the total concentration of thyroxine in euthyroid tissues of the rat. The swelling is ordinarily measured by following the decrease in optical density of the suspension; it has been found that this decrease corresponds to a gain in water content (Lehninger¹⁵).

In the usual medium of 0.30 M sucrose the swelling action of thyroxine is characterized by a bell-shaped pH rate curve with a single maximum at pH 7.5; no swelling activity is evident at pH 6.5 or at pH 8.5.¹¹ Thyroxine-induced swelling is characterized by a short lag period followed by a pseudo first-order fall to a limiting lower limit of optical density corresponding almost to a threefold increase in water content.¹¹ The rate of swelling has an extra-

ordinarily high temperature coefficient and is extremely rapid at 37° C.¹¹ Similar properties are observed when the test medium is 0.15 *M* KCl or NaCl, although the mitochondria are far more sensitive to thyroxine when in KCl than in sucrose-containing media. In fact, increasing the sucrose concentration can abolish the thyroxine-induced swelling.^{11,15}

The swelling induced by thyroxine can be prevented not only by the presence of dinitrophenol, as mentioned before, but also by Mg^{++} , ATP, ethylenediaminetetraacetic acid tetrasodium salt (EDTA), and by small amounts of bovine serum albumin, but not by equivalent concentrations of other proteins.^{4,11} These agents also inhibit or prevent swelling induced by phosphate or Ca^{++} ; these actions of Mg^{++} and ATP, as well as that of DNP, are suggestive of a relationship between the action of swelling agents and the energy-coupling mechanisms of oxidative phosphorylation.

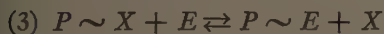
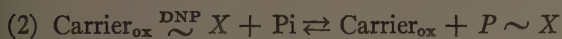
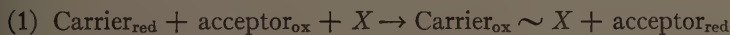
Still further evidence for an enzymatically controlled process as the target of the swelling agents came from studies on the respiratory inhibitors. The striking observation was made that the swelling action of thyroxine¹⁶ and of phosphate^{16,17} is completely abolished by anaerobiosis or by the addition of cyanide. It was soon found^{11,18} that the swelling action of thyroxine is also abolished by the respiratory inhibitors antimycin A and sodium Amytal, which inhibit respiration at different levels in the chain. Since bound diphosphopyridine nucleotide (DPN) is lost when mitochondria are exposed to thyroxine,^{11,19} it was at first suggested that maintenance of mitochondrial DPN in the reduced form by inhibition of electron transport²⁰ was the factor responsible for failure of the mitochondria to swell¹¹ in the presence of respiratory inhibitors. However, this interpretation now seems to be incorrect in view of the work of Chappell and Greville²¹ and of Hunter *et al.*,¹⁸ who have shown clearly that mitochondrial swelling cannot occur unless some respiration is taking place. Although the addition of respiratory substrates is ordinarily not made in such experiments, the mitochondria have significant endogenous respiration. Inhibition of swelling by the respiratory inhibitor amytal (which inhibits at the DPN-flavoprotein step and thus keeps DPN in the reduced state) can be relieved by succinate, which can restore respiration beyond the point of Amytal inhibition. The effect of succinate can be prevented, in turn by the addition of malonate. This decisive experiment, as well as the finding that the swelling response can be restored in aged mitochondria by adding respiratory substrates such as β -hydroxybutyrate, provides very good evidence that some endogenous electron transport must occur for mitochondrial swelling to take place.

What physical changes take place when thyroxine induces swelling? Recent experiments on mitochondrial permeability show that, although fresh mitochondria contain a water compartment amounting to about 60 per cent of the mitochondrial volume, which is easily penetrated by sucrose at 0° C., on swelling or aging nearly 100 per cent of the mitochondrial water becomes penetrated by sucrose.²² These findings suggest the existence of 2 concentric morphological compartments, each delineated by one of the mitochondrial membranes, of which the inner is normally impermeable to sucrose.¹¹ This construct implies that thyroxine (or other swelling agents) increases the permeability of the inner compartment to sucrose. This implication has in fact been established¹¹ by the finding that once thyroxine-induced swelling has begun it cannot be stopped

or reversed by increasing the concentration of sucrose in the medium, whereas the addition of a high-molecular-weight solute such as polyvinylpyrrolidone can reverse the swelling, presumably because it cannot pass the membrane.

The evidence summarized above thus indicates clearly that mitochondrial swelling induced by thyroxine is not a simple or passive process. Mitochondrial swelling requires continuous electron transport, and evidently at least a portion of the energy coupling mechanisms must be intact and functioning, since the uncoupling agents dinitrophenol or Dicumarol inhibit swelling. Coupled phosphorylation of adenosine diphosphate (ADP) may or may not be necessary also; lowered intramitochondrial ATP may be involved in the susceptibility to swelling,²³ and addition of ADP or ATP *in vitro* causes inhibition of swelling induced by thyroxine.⁴

Recent work²⁴⁻²⁷ on the mechanism of oxidative phosphorylation suggests the following pattern:



Three such complex processes occur at 3 points in the respiratory chain. The respiratory chain enzymes, as well as the coupling enzymes, are entirely present in the membranes and, indeed, make up a large part of its substance.²⁴ Changes in the activity of these enzymes thus could cause changes in the mechanical properties of the membranes. It may be presumed that the portion of the coupling mechanism that lends the mitochondrial structure susceptibility to thyroxine (or phosphate, Ca^{++} , or glutathione [GSH]) lies between the point of action of DNP as shown and the final product ATP, since DNP and ATP¹¹ each prevent thyroxine-induced swelling. However, no clear deduction is possible as to the component involved in changing permeability from studies of inhibitors. For example, azide has been shown to inhibit coupling between the point of DNP action and Reaction 4, possibly at Reaction 3,²⁵ but azide prevents thyroxine-induced swelling.⁴ It must be recalled again that thyroxine (or its simpler analogues) does not uncouple phosphorylation in subfragments of mitochondria, nor has it been observed to inhibit partial reactions of this scheme insofar as they are now measurable.²⁷ The observation of Bronk²⁸ that thyroactive compounds have significant effects on coupling in sonic subfragments prepared by a procedure different from that referred to above³ may mean only that such particles retain more structure and thus are more nearly similar to intact mitochondria than the digitonin fragments.

It must be supposed, therefore, that some element of the mitochondrial membrane structure depends on or is in equilibrium with one of the separate reactions of the coupling sequence above. For example, it could be visualized that the intermediate $P \sim E$ above, while not directly reactive to the four swelling agents, may stand in equilibrium with an enzyme of the membrane not itself in the coupling scheme, but one that contributes crucially to the structure and permeability of the mitochondrial membrane and is susceptible

to the swelling agents, as follows:



Such a protein M could be visualized as a "mechanoenzyme" capable of changing shape and thus properties of the membrane, depending on whether it is phosphorylated or not.¹⁵ Maintenance of M in the phosphorylated form, for example, would keep the membrane contracted. It could be postulated that thyroxine can interfere in the formation of $P \sim M$ from $P \sim E$ and thus cause swelling.

There is also some evidence that the target of the action of thyroxine may be mitochondrial DPN, since the loss of thyroxine susceptibility of mitochondria on aging correlates with the loss of mitochondrial DPN.¹¹ Mitochondrial DPN exists in bound forms (possibly of a high-energy nature) that may have specific functions in oxidative phosphorylation.^{24,29} ATP is capable of "rebinding" mitochondrial DPN.³⁰ It is of interest that thyroxine is capable of inhibiting a number of DPN-linked dehydrogenases containing metal, but not triose phosphate dehydrogenase,³¹ thus suggesting that it may be a "bifunctional" inhibitor.

The contraction of thyroxine-swollen mitochondria. Although thyroxine-induced swelling in sucrose media can be prevented readily by agents such as ATP, Mg^{++} , EDTA, and DNP, these agents do not contract the mitochondria once they have swollen. Very high concentrations of high-molecular-weight solutes such as serum albumin or polyvinylpyrrolidone do contract or shrink the mitochondria;¹¹ in such experiments the mitochondria contract "passively" as would any structure, dead or alive, surrounded by a membrane impermeable to these substances. The failure until recently to observe "active" or specific contraction of thyroxine-swollen mitochondria loomed as quite embarrassing to any view that this effect of thyroxine represents its mode of action. This fact prompted further study of mitochondrial contraction.

Earlier studies by Price *et al.*³² and also by Ernster and his colleagues^{23,33} showed that swollen mitochondria could be induced to contract if respiration were instituted. Also, Chappell and Perry had observed a low degree of contraction of muscle mitochondria by the addition of ATP alone.³⁴ However it was clearly evident when we repeated these studies that some factor operated against reproducible observation of mitochondrial contraction. The situation was clarified to a great extent when it was found that mitochondrial contraction induced by thyroxine could be very easily and completely reversed by the simple addition of ATP, providing the medium did not contain sucrose.^{11,15,35} Sucrose inhibits not only mitochondrial swelling but also mitochondrial contraction. If thyroxine-induced swelling is carried out in a medium of NaCl or KCl, or even in distilled water alone, then the simple addition of ATP contracts the mitochondria with extrusion of water, which was measured gravimetrically. From such observations we have postulated that sucrose is efficacious in isolating and preserving mitochondria because it inhibits some factor involved in the swelling and contraction cycle and thus "freezes" the morphology of these bodies.^{11,15} It is significant that sucrose also uncouples phosphorylation and inhibits an intermediate reaction in oxidative phosphorylation.^{11,36}

The contraction of thyroxine-swollen mitochondria has been found to be

specific for ATP; other triphosphates and ADP are inactive.¹⁵ However, ADP has some activity in contraction of hypotonic mitochondria,³⁷ but this is a consequence of the action of adenylate kinase, since fluoride inhibits this effect of ADP without affecting contraction by ATP. The contraction process is remarkably stable; thyroxine-induced mitochondria can be allowed to remain in the swollen state for hours at room temperature and still contract promptly on addition of ATP, with extrusion of water. This observation led to the finding that contraction of swollen mitochondria by ATP does not depend on an intact and functioning respiratory chain or on an intact coupling mechanism, since it was found that the contraction by ATP proceeds maximally in the presence of cyanide or antimycin A and is not inhibited by dinitrophenol. Azide, however, inhibits the contraction. The contraction process thus differs significantly from the swelling phenomenon, which requires a functional respiratory chain.

ATP with adjuvants of Mg^{++} and serum albumin is able to contract mitochondria swollen by almost every swelling agent known.³⁷ Thus, we have observed that ATP shrinks mitochondria swollen by thyroxine, Ca^{++} , glutathione, phosphate, U factor,^{37,38} fatty acids, *p*-chloromercuribenzoate, hypotonicity, digitonin, phloridizin,³⁹ and other agents.

It is interesting to compare the amounts of water extruded from thyroxine-swollen mitochondria with the amount of ATP added or split. It has been found that many moles of H_2O may be extruded per mole of ATP added, and several hundred moles of H_2O per mole of ATP split.¹⁵ Ratios as high as 2400:1 have been observed. It is of some interest that ATP hydrolysis ceases when contraction stops, suggesting a relationship between ATP splitting and the state of contraction of mitochondria.¹⁵

Recently we have found that a soluble protein that escapes from swollen mitochondria is necessary in the contractile process.⁴⁰ If GSH-swollen mitochondria are recovered by centrifugation and placed in a fresh salt medium, they will not contract in the presence of ATP, but will do so if supplemented with a heat-labile, nondialyzable component separated from the medium in which the swelling had taken place. This protein is possibly enzymatic in nature, and it is now being purified.

Mitochondrial contraction seems clearly to be an enzymatic process on the basis of the specificity of ATP and the inhibitory action of sucrose and azide. Indeed, these properties suggest a mechanistic relationship of contraction to oxidative phosphorylation, as is the case for swelling. While it appears quite possible that an intermediate reaction of oxidative phosphorylation is also shared by both the swelling and contraction processes (the azide-sensitive and sucrose-sensitive reactions, for example), it is at present not at all certain that swelling and contraction are reflections of a single reversible process. Contraction may proceed in the absence of electron transport (which is necessary for swelling) and is not affected by DNP (which inhibits swelling); these facts seem incompatible with a simple reversible reaction if current views of the mechanism of energy coupling are correct. On the other hand it appears not unreasonable to suggest that swelling and contraction are not only enzymatically but also morphologically distinct reactions. The assumptions required are that the outer membrane is the site of contractile activity with ATP and is freely per-

meable to most small solutes either in the relaxed or contracted state. It is also necessary to suppose that the inner membrane is the site of those reactions leading to swelling and increase of permeability toward sucrose (and thus the observed increase in penetration of mitochondrial water by sucrose). The inner membrane is also believed to be the site of the respiratory carriers. On this basis the outer membrane, activated by ATP, should be able to contract the mitochondrion, regardless of the condition of the inner membrane. The known ability of ATP to prevent any kind of swelling and its ability to reverse any kind of swelling could thus be explained. However, the inhibition of contraction, as well as swelling, by azide and sucrose remains as a significant deterrent to accepting this explanation fully.

Regardless of the relationship of swelling and contraction, a mechanoenzyme basis for contraction¹⁵ as well as swelling seems attractive. The phosphoprotein of mitochondria, it has been suggested, may be a possible element in an ATP-driven contractile mechanism¹⁵ similar in principle to the actomyosin system of muscle.

With this summary of the properties of mitochondrial swelling and contraction as a basis, we may consider other questions. What is the relationship of mitochondrial swelling to uncoupling of phosphorylation? Does swelling occur as the first symptom of the action of thyroxine on mitochondria, which is then followed by frank uncoupling of phosphorylation, or do swelling and uncoupling occur simultaneously? In liver mitochondria it appears certain that mitochondrial swelling may take place before significant uncoupling occurs. We have found that the $P:2e$ ratio of the oxidation of β -hydroxybutyrate by liver mitochondria does not drop appreciably in the first one third to one half of the thyroxine swelling curve. However, during this period DPN is lost from the mitochondria. Also significant is the finding that the respiration of the mitochondria is changed from "tightly coupled" to "loosely coupled" respiration in the early phase of swelling; that is, respiration loses the dependence on the presence of phosphate and ADP that is so characteristic of tightly coupled mitochondria. However, in this change there is no significant decline in the $P:2e$ ratio. This finding may be a reflection of the activity of "R factor," a protein-containing fraction of mitochondria which has been found to loosen the coupling between phosphorylation and respiration without impairing the $P:2e$ ratio significantly.⁴¹ It will be recalled that Hoch and Lipmann had reported the incidence of loose coupling in mitochondria isolated from hyperthyroid animals.⁴² The activation or release of R factor may thus be responsible for respiratory changes accompanying early stages of swelling.

When swollen mitochondria are allowed to stand in contact with thyroxine for more extended periods, not only is most or all of the DPN lost, but the ability to carry out oxidative phosphorylation is almost completely lost. Thus, a sequence of symptoms results from exposure of liver mitochondria to thyroxine, of which the first detected is induction of loose-coupling, without significant lowering of phosphorylation efficiency.

The specificity of thyroxine and similar molecules in causing mitochondrial swelling has been examined (Lehninger, unpublished experiments). A survey of about 30 compounds has revealed that all of those substances tested that are active in the B.M.R. or metamorphosis assays are also active in promoting

swelling, and the activities are roughly proportional. Significantly, swelling activity is substantially greater in those derivatives of thyroxine in which the alanyl side chain is replaced by acetic, propionic, or acrylic residues, the latter replacement increasing activity five- to tenfold. On the other hand, D-thyroxine is as active as L-thyroxine. However, the work of Freinkel *et al.*⁴³ has shown that D-thyroxine is more rapidly metabolized than the L isomer and its apparent lack of thyroactivity in the mammal may be caused by its failure to remain at the target site at a sufficiently high concentration for a period adequate to evoke a response.

An observation has been made that is of interest in connection with the difference in action between thyroxine and triiodothyronine on the one hand and their acetic acid analogues on the other. It has been found that the acetic acid analogues have essentially no lag period in inducing swelling and that they also cause rapid swelling at low temperatures (10–15° C.), whereas thyroxine and triiodothyronine both have significant lag periods that are greatly increased at low temperatures.¹¹

While I have not yet found any significant inhibitory action of thyroactive compounds on intermediate or partial reactions of oxidative phosphorylation, these may yet be found to occur on subtle variations of conditions, particularly since certain phosphate-transferring enzymes such as acylphosphatase⁴⁴ and carbamylphosphatase⁴⁵ activities seem to be quite sensitive to the hormone. Indeed, the observations of Harary⁴⁴ on uncoupling of the glycolytic cycle by thyroxine, already of great intrinsic interest as they stand, may also serve as a prototype or model of the uncoupling action of thyroxine on oxidative phosphorylation.

In evaluating the physiological significance of the swelling action of thyroxine on mitochondria it must be remembered that swelling is but one of several significant effects of the hormone. Attention is called to the significant work of Phillips and Langdon,⁴⁶ who have shown that microsomal triphosphopyridine nucleotide (TPN)-cytochrome *c* reductase activity is greatly increased in hyperthyroid rats, and also the more recent work of Lardy *et al.*,⁴⁷ who have demonstrated a sevenfold increase of mitochondrial α -glycerophosphate dehydrogenase on feeding thyroid to rats. These observations indicate that thyroxine may control the balance of extramitochondrial oxidized and reduced pyridine nucleotides (important in biosynthetic reactions) by regulating oxidation via mitochondrial and microsomal pathways. These effects of thyroxine may in fact be related to its action on mitochondria. It will be recalled that fresh, intact mitochondria do not oxidize extramitochondrial DPNH.⁴⁸ Reducing equivalents furnished by external DPNH may, however, be oxidized in mitochondria either by the so-called " α -glycerophosphate shunt"⁴⁷ or by the β -hydroxybutyrate-acetoacetate "shuttle."⁴⁹ It is significant that swelling of the mitochondria can increase the rate of the latter mechanism, presumably by increasing the accessibility of DPNH.⁵⁰

Perhaps the most convincing evidence for a physiological role of thyroxine in controlling the state of swelling of mitochondria *in vivo* is provided by two other lines of evidence. It was found by Tapley⁴ in our laboratory that mitochondria isolated from hyperthyroid rat livers had a much higher spontaneous swelling rate than did normal mitochondria; those from hypothyroid rats, on

the other hand, were very resistant to spontaneous swelling. These properties were entirely consistent with expectations from experiments on *in vitro* addition of thyroxine to mitochondria. Of most significance is the finding of Sjöstrand and his colleagues, by electron microscopy, that the mitochondria of hyperthyroid tissues are distinctly swollen and show diffuse cristae.⁵¹

Because it is now becoming clear that epinephrine is involved in an important way in the action of the thyroid secretion, it will be important to examine more closely evidence of interaction between epinephrine and other catecholamines and thyroxine in mitochondrial swelling and contraction, as well as in the efficiency of phosphorylation and the "tightness" of respiratory coupling. Such studies are now under way.

Summary

Evidence has been summarized to show that the swelling of mitochondria requires the occurrence of electron transport and is promoted by thyroxine, as well as by phosphate, Ca^{++} , and reduced glutathione. Contraction of mitochondria, with accompanying extrusion of water, occurs in the presence of ATP. Contraction, however, requires neither electron transport nor complete integrity of the energy-coupling enzymes, but is characteristically inhibited by azide and sucrose.

The first detectable symptoms of the swelling action of thyroxine on mitochondria are the gradual loss of bound DPN and the induction of "loose-coupling" of respiration, which may occur by the action of R-factor. Following more drastic swelling by thyroxine, the permeability to sucrose increases. The swelling activity of thyroxine and its analogues follows, in general, their activity in B.M.R. and metamorphosis tests. Some evidence exists that, in hyperthyroidism, mitochondria of some tissues are swollen *in vivo*.

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THE INFLUENCE OF THYROXINE AND RELATED COMPOUNDS ON OXIDATIVE RATE AND EFFICIENCY OF PHOSPHO- RYLATION IN LIVER MITOCHONDRIA AND SUB- MITOCHONDRIAL PARTICLES*

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Introduction

Many groups of investigators¹⁻³ have made the observation that thyroxine will uncouple oxidative phosphorylation in mitochondria provided that the mitochondria have been pretreated in one of a variety of ways. Such uncoupling has generally not resulted in an increased rate of respiration by the isolated mitochondria, but, as Lardy *et al.* have pointed out, a lower efficiency of phosphorylation would necessitate increased respiration by the whole cell in order to provide the same amount of energy for the various cellular activities. In consideration of the relatively high concentration of thyroxine required, Lehninger and his colleagues³ have expressed the view that the uncoupling of oxidative phosphorylation by thyroxine is more likely to be related to the action of the hormone in thyrotoxicosis than in the normal state.

This paper describes some experiments I have carried out that demonstrate direct effects of thyroxine and some of its analogues on the process of oxidative phosphorylation in fragments of mitochondria. These fragments were prepared using sonic disintegration of rat liver mitochondria. Obviously, the preparation is a highly unnatural one; therefore, strong reservations must be made in any attempt to draw parallels between the effects of thyroxine on this preparation and its influence *in vivo*. Nevertheless, it is possible to relate the results that have been obtained with submitochondrial particles to the influence of thyroxine on whole mitochondria. Furthermore, the nature of some of the observed actions of thyroxine is at least consistent with some of its effects on intact animals.

Materials and Methods

The submitochondrial particles were prepared by the method previously described by Kielley and myself.⁵ The important features of this method are the suspension of the mitochondria in hypotonic phosphate buffer (0.03 M), treatment in the sonic oscillator for only 30 sec. and, finally, isolation of the particles that sediment between 25,000 and 100,000 g. Particles prepared in this manner will oxidize succinate or reduced diphosphopyridine nucleotide (DPNH). In the presence of 5 μ M/ml. adenosine diphosphate (ADP), 5 μ M/ml. of $MgCl_2$, and 10 μ M/ml. inorganic phosphate at pH 7.0, this oxidation will be accompanied by net synthesis of adenosine triphosphate (ATP). The addition of cationic diphosphopyridine nucleotide (DPN^+) is essential for the oxidation of β -hydroxybutyrate by the particles, and the preparation does not appear to contain any dehydrogenases other than succinic and β -hydroxy-

* A portion of the experimental work reported in this paper was made possible by a grant from the Public Health Service, Bethesda, Md.

butyric dehydrogenase. Also, the particles are virtually free of myokinase activity. In addition to oxidative phosphorylation, the particles will catalyze an ATP- P^{32} exchange, an ATP-ADP 32 exchange, and ATPase. Previously published studies⁵ have revealed that all of the phosphorylation catalyzed by the particles is associated with the steps of the electron-transport chain prior to the level of cytochrome *c*. No phosphorylation is associated with electron transport from cytochrome *c* to oxygen. Thus the maximum P/O ratio that is obtainable with succinate is 1.0 and, with DPNH or β -hydroxybutyrate plus DPN⁺, is 2.0. In practice, the P/O ratios vary considerably from one preparation to another and are often far below these maximum values. This result is probably due to the presence of some natural uncoupling agent such as that recently described by Lehninger and Remmert.⁶

Mention also must be made of the method used to measure oxygen uptake. This was estimated, using the Clarke oxygen electrode. When employed with a suitable polarizing circuit and a recording device, this instrument provided a continuous recording of the oxygen tension in the incubation vessel. Using an incubation volume of 1.9 ml. it was possible to measure accurately an oxygen uptake as small as 0.05 μ atoms of oxygen. The usual practice was to continue the incubation until at least 0.2 to 0.3 μ atoms of oxygen had been consumed. This corresponded to roughly 30 per cent of the oxygen dissolved in 1.9 ml. at 28° C. and meant that the incubation time could be reduced to about 2 min. Phosphate uptake was measured by estimation of the incorporation of P^{32} into ATP, as previously described.⁵

Results and Discussion

The influence of thyroxine on oxidative phosphorylation in submitochondrial particles. Using these methods it has been possible to demonstrate, as I have indicated elsewhere,^{7,8} that there are 3 effects of thyroxine on the submitochondrial particles. FIGURE 1 shows that thyroxine caused an increase in the initial rate of oxygen uptake, with succinate as the substrate. The dotted lines indicate oxygen uptake in the control incubations, while the solid lines show the oxygen uptake in the presence of $2.5 \times 10^{-5} M$ thyroxine. In all cases the particles were preincubated with the reaction medium for 30 sec. prior to the addition of the substrate. The 2 lower curves represent the oxygen uptake obtained with a 1 to 2.5 dilution of the submitochondrial particle suspension and indicate that thyroxine has a greater influence on the oxygen uptake of the more dilute preparation.

TABLE 1 shows that there was also an improvement in P/O ratio associated with the stimulation of oxygen uptake that occurs in the presence of thyroxine. In addition to the rates of oxygen uptake and the P/O ratios, the percentages of the control values obtained with thyroxine present are indicated.

Four different preparations are represented in TABLE 1, and it is clear that there was considerable variability in the extent of their effects. The stimulation of oxygen uptake by thyroxine and the accompanying improvement in P/O ratio were generally of the order of 20 to 50 per cent, with succinate as the substrate although, as the data obtained with preparation No. 3 indicated, stimulations as high as 100 per cent were sometimes found. It is also clear that in the presence of thyroxine the P/O ratios tended toward 1.0, but never

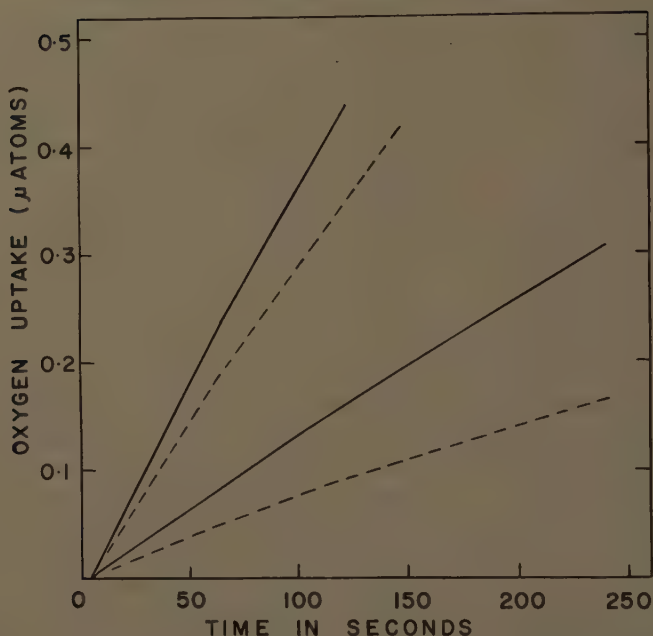


FIGURE 1. The time course of oxygen uptake by submitochondrial particles. *Dotted lines*, controls; *solid lines*, incubations in the presence of $2.5 \times 10^{-5} M$ thyroxine. *Upper curves*, 0.2 mg. *N*; *lower curves*, 0.08 mg. *N*. Other conditions as in TABLE 1.

TABLE 1
THE INFLUENCE OF THYROXINE ON THE OXIDATION OF SUCCINATE
AND ASSOCIATED PHOSPHORYLATION

Preparation	Thyroxine present (final concentration)	Oxygen uptake		P/O ratio	
		μatoms O/min.	Per cent control		Per cent control
No. 1	None	0.081	—	0.42	—
	$2.5 \times 10^{-5} M$	0.107	132	0.54	129
No. 2	None	0.127	—	0.28	—
	$2.5 \times 10^{-5} M$	0.156	123	0.37	132
No. 3	None	0.077	—	0.32	—
	$2.5 \times 10^{-5} M$	0.150	195	0.62	194
No. 4	None	0.134	—	0.41	—
	$5 \times 10^{-5} M$	0.209	156	0.61	149
(Hexokinase added)	None	0.150	—	0.62	—
(Hexokinase added)	$5 \times 10^{-5} M$	0.222	148	0.77	124

Conditions: 10 μM ADP, 10 μM adenosine monophosphate (AMP), 10 μM $MgCl_2$, 20 μM phosphate, pH 7.0 (10^6 cpm P^{32}), 5 μM succinate; Preparation No. 1, 0.21 mg. *N*; No. 2, 0.13 mg. *N*; No. 3, 0.11 mg. *N*; No. 4, 0.19 mg. *N*; where hexokinase was added, 50 μM glucose were present. Final volume 1.9 ml.; temperature 28° C. Incubation time, 75 to 180 sec., giving 0.2 to 0.3 μatoms of oxygen consumed. Submitochondrial particles added to incubation medium 30 sec. prior to addition of substrate.

exceeded it. A P/O ratio of 1.0 is theoretically the maximum obtainable using the submitochondrial particles with succinate as the substrate. The last 2 incubations in TABLE 1 indicate that the addition of hexokinase and glucose to trap the ATP formed did not alter the basic nature of the thyroxine effects.

Similar, although somewhat less striking, effects of thyroxine were observed when DPNH or β -hydroxybutyrate were used as substrates (TABLE 2). Once again, the rate of oxygen uptake and the P/O ratio were greater in the presence of thyroxine although, as the last 4 incubations in TABLE 2 show, the rate of oxidation of β -hydroxybutyrate was stimulated by thyroxine only in the presence of excess DPN⁺. This fact, together with the observed stimulation of DPNH oxidation, indicate that thyroxine was stimulating the oxidation of DPNH rather than the activity of the β -hydroxybutyric dehydrogenase. In fact, I have observed inhibition of the dehydrogenase by higher concentrations of thyroxine—a type of effect first described by Wolff and Wolff.⁹

TABLE 2
THE INFLUENCE OF THYROXINE ON OXIDATIVE PHOSPHORYLATION
WITH DPNH AND β -OH BUTYRATE AS SUBSTRATES

Preparation	Substrate	Thyroxine present (final concentration)	Oxygen uptake		P/O ratio	
			μ atoms O/min.	Per cent control		Per cent control
No. 1	DPNH	None	0.299	—	0.41	—
No. 2	DPNH	$2.5 \times 10^{-5} M$	0.372	124	0.53	129
	DPNH	None	0.376	—	0.63	—
	DPNH	$5 \times 10^{-5} M$	0.470	125	0.77	118
	β -OH butyrate + 1 μ M DPN ⁺	None	0.137	—	0.84	—
	β -OH butyrate + 1 μ M DPN ⁺	$5 \times 10^{-5} M$	0.145	106	0.94	112
	β -OH butyrate + 2 μ M DPN ⁺	None	0.214	—	0.92	—
	β -OH butyrate + 2 μ M DPN ⁺	$5 \times 10^{-5} M$	0.261	121	1.02	111

Conditions: where used, 2.5 μ M DPNH or 10 μ M β -hydroxybutyrate; preparation No. 1, 0.19 mg. *N*; No. 2, 0.18 mg. *N*; other conditions as in TABLE 1.

The upper two curves of FIGURE 2 indicate the relationship between thyroxine concentration and the percentage stimulation of oxygen uptake and P/O ratio with succinate as the substrate. All of the incubations represented in this figure were performed with a single particle preparation. The data presented above were all obtained with thyroxine concentrations of $2.5 \times 10^{-5} M$ or $5 \times 10^{-5} M$, but some stimulation of rate of oxygen uptake and P/O ratio was obtained with thyroxine concentrations as low as $5 \times 10^{-6} M$, as shown by FIGURE 2.

The lower 2 curves in FIGURE 2 illustrate the third effect of thyroxine that can be demonstrated with the submitochondrial particles. If the particles were preincubated in the reaction medium for 2 min. in the absence of substrate and if, at the end of this preincubation period, thyroxine was added, followed in 30 sec. by the substrate, the results shown in the lower 2 curves were obtained. At concentrations of $5 \times 10^{-5} M$ and above, thyroxine markedly inhibited phosphorylation under these circumstances. These data show that a higher

concentration of thyroxine was required for the inhibition of phosphorylation than for the stimulation of oxygen uptake and the improvement in P/O ratio; also that the inhibition of phosphorylation was not accompanied by any significant rise in the oxidative rate. The inhibition of phosphorylation occurred only when the submitochondrial particles had been preincubated with the reaction medium for about 2 min. prior to the addition of the thyroxine. Pre-

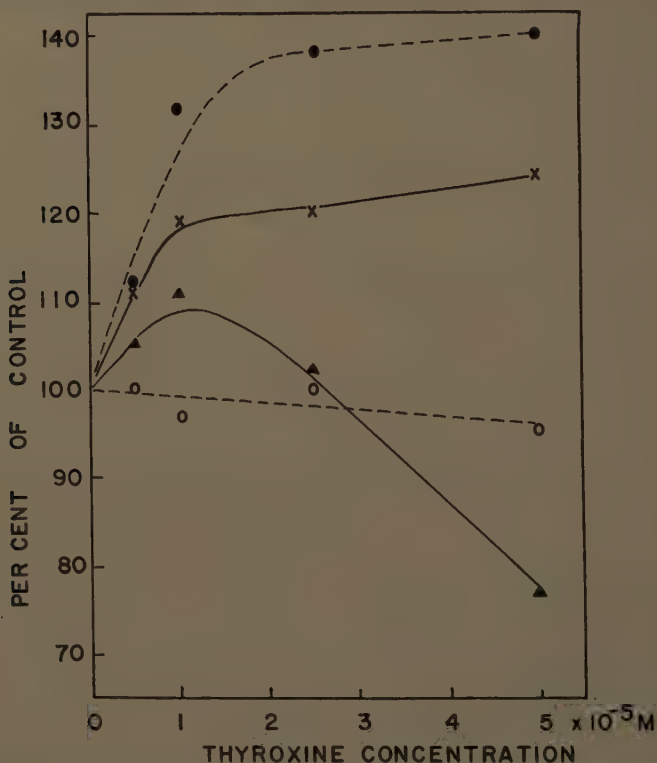
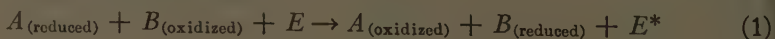
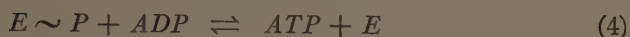
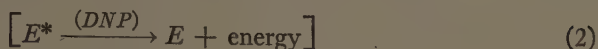


FIGURE 2. The influence of thyroxine concentration on rate of oxygen uptake and P/O ratio in submitochondrial particles. Symbols: ●---●, oxygen uptake; ×—×, P/O ratio; ○---○, oxygen uptake after preincubation for 2 min.; ▲—▲, P/O ratio after preincubation for 2 min. All points were obtained by taking the percentage of control value in the absence of thyroxine. Five μ M succinate as substrate; 0.21 mg. *N*; other conditions as in TABLE 1.

incubation in the presence of thyroxine, on the other hand, produced the usual stimulation of oxygen uptake and improved P/O ratio.

Evidence for the point of action of thyroxine on oxidative phosphorylation. The following sequence of reactions is a vastly oversimplified scheme for the process of oxidative phosphorylation. Here *A* and *B* represent 2 consecutive members of the electron transport chain; *E* is an intermediate that can exist in a normal or a high-energy state (the latter being denoted by E^*); *P* or P_i are used to denote inorganic phosphate.





In REACTION 1 I have grouped together the steps concerned with the transfer of electrons from one member of the electron transport chain to the next and the coupled formation of a high energy intermediate E^* in this formulation. REACTION 1 undoubtedly occurs in 2 or more steps, and the exact mechanism involved is not known. REACTIONS 3 and 4 represent the terminal steps of the phosphorylation process and are concerned with the transfer of the extra energy present in the E^* intermediate to form the terminal high-energy phosphate bond of ATP. Evidence for the involvement of inorganic phosphate before ADP has been published by Kielley and myself.¹⁰

REACTION 2, which would not be present in a tightly coupled system, is present in the submitochondrial particles due to the action of some natural uncoupling agent. REACTION 2 can be stimulated by 2,4-dinitrophenol (DNP) and accounts for the ATPase activity of the particles and for their relative lack of respiratory control. As E^* is formed by REACTION 1, it is partitioned between REACTIONS 2 and 3, and this is responsible for the fact that the observed P/O ratios were lower than the theoretical ones.

The possible points at which thyroxine could act on this mechanism in order to cause an improvement in P/O ratio are: stimulation of REACTION 3, inhibition of REACTION 2 or, under certain circumstances, stimulation of REACTION 1. Stimulation of REACTION 4 would not be expected to influence the P/O ratio, since previously published data¹⁰ have indicated that the rate of REACTION 4 is much greater than the rate of REACTION 3.

In order to decide between these various possibilities, experiments were performed to test the influence of thyroxine on the ATP- P^{32} exchange reaction (represented by REACTIONS 3 and 4) and ATPase (represented by REACTIONS 4, 3, and 2 in that order). Thyroxine was found to cause no alteration in the rate of either process⁸ and, therefore, presumably none of the last 3 steps is influenced by thyroxine. By a process of elimination we are thus left with REACTION 1 as the site of thyroxine action in the submitochondrial particles. According to the scheme shown here, thyroxine could influence REACTION 1 in at least 2 different ways that could result in higher P/O ratios. The simplest effect would be merely an increase in the rate of REACTION 1. This would result in a higher rate of formation of E^* and, if we make the not unreasonable assumption that the rate of REACTION 2 is limited by the amount of natural uncoupling agent present, then more E^* per min. would produce a higher P/O ratio. A second possible mode of action of thyroxine on REACTION 1 would be to increase the efficiency of the energy transfer that occurs in the series of steps represented by this reaction. Either of these 2 alternatives would result in an increased rate of oxygen uptake as well as an improved P/O ratio, thereby eliminating the necessity for postulating 2 separate thyroxine effects to account for the observed stimulation of oxidation rate and P/O ratio.

At the moment, the experimental evidence is insufficient to decide in favor of either of these 2 possibilities. If the first one were to apply, it would mean

that thyroxine acted primarily to increase the rate of electron transport, and that the influence of thyroxine on P/O ratio was due merely to the presence of REACTION 2 in the submitochondrial particles. The second possibility would require that the primary effect of the thyroxine be on the coupling process, the increased oxidative rate being due to an increased degree of coupling.

Neither of the possible modes of action of thyroxine just mentioned could easily be used to explain the inhibition of phosphorylation that results from the addition of thyroxine to the submitochondrial particles after a preincubation period. The inhibition presumably requires some sort of change in the particle preparation that is not understood at present. More must be known about what happens to the particles during the preincubation period.

The influence of thyroxine on oxidative phosphorylation in intact mitochondria. Some notion of how physiological the effects of thyroxine on the submitochon-

TABLE 3

THE INFLUENCE OF THYROXINE ON OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA

Thyroxine present (final concentration)	MgCl ₂ added (μ moles)	Preincubation (min.)	Oxygen uptake		P/O ratio	
			μ atoms O/min.	Per cent control		Per cent control
0	0	0	0.402	—	1.45	—
2.5×10^{-5} M	0	0	0.415	103	1.55	107
0	0	2	0.330	—	1.33	—
2.5×10^{-5} M	0	2	0.302	92	0.71	53
0	10	0	0.385	—	1.44	—
2.5×10^{-5} M	10	0	0.389	101	1.50	104
0	10	2	0.304	—	1.47	—
2.5×10^{-5} M	10	2	0.342	112	1.51	103

Conditions: 2 μ M ADP, 15 μ M AMP, 5 μ M succinate, 20 μ M phosphate, pH 7.0 (10^6 counts/min. P^{32}), 200 μ M sucrose, 0.65 mg. protein *N*. Total volume 1.9 ml., temperature 28° C. Time of incubation, 45–75 sec., giving 0.4–0.5 μ atoms of oxygen consumed.

drial particles are can be obtained by comparing them with the effects of thyroxine on intact mitochondria. As the top 2 incubations in TABLE 3 indicate, in confirmation of the results of others,³ thyroxine had little or no influence on the rate of oxidation or P/O ratio when intact mitochondria maintained in isotonic sucrose were tested with succinate as the substrate. If, however, the mitochondria were preincubated in the presence of thyroxine until the endogenous substrate was used up (about 2 min. under these conditions), and then the substrate was added, the usual inhibition of phosphorylation was observed; the control was virtually unaffected. Apparently some change was required in intact mitochondria as well as in submitochondrial particles in order to make it possible for thyroxine to inhibit phosphorylation. As was the case with the submitochondrial particles, there was no increase in oxidation rate associated with the lower P/O ratio of the mitochondria preincubated with thyroxine. However, as shown in the lower half of TABLE 3, the inhibition of phosphorylation in intact mitochondria was reversed by the addition of magnesium chloride, unlike the inhibition of phosphorylation by thyroxine in submitochondrial particles. Therefore, the inhibition of phosphorylation by thyroxine following

preincubation of mitochondria was not due to the same mechanism as the inhibition of phosphorylation that followed preincubation of the submitochondrial particles.

As long as mitochondria were maintained in isotonic sucrose there was no evidence of any stimulation of oxygen uptake or P/O ratio by thyroxine. Radical treatment of the mitochondria, such as washing them in hypotonic phosphate buffer (0.03 *M*), caused a drop in the rate of oxygen uptake and a lower P/O ratio, as shown in TABLE 4. The addition of thyroxine to the phosphate-washed mitochondria caused a marked increase in the rate of oxygen uptake and did not reduce the P/O ratio, since magnesium chloride was present. This experiment may provide some insight into the stimulation of oxygen uptake by thyroxine observed with the submitochondrial particles, since the first step in producing the particles was to wash the mitochondria in hypotonic phosphate buffer.

TABLE 4
THE INFLUENCE OF THYROXINE ON NORMAL AND SWOLLEN MITOCHONDRIA

Suspension medium	Thyroxine present (final concentration)	Oxygen uptake		P/O ratio	
		μ atoms O/min.	Per cent control		Per cent control
Isotonic sucrose	None	0.389	—	1.54	—
Isotonic sucrose	5×10^{-5} <i>M</i>	0.380	98	1.66	108
Hypotonic phosphate	None	0.180	—	1.18	—
Hypotonic phosphate	5×10^{-5} <i>M</i>	0.269	150	1.20	102

Conditions: The same as TABLE 3 except that 10 μ M of $MgCl_2$ were present in all incubations and, when mitochondria suspended in hypotonic phosphate were used, the 200 μ M of sucrose were omitted from the incubation medium; 0.61 mg. *N*.

Intact mitochondria maintained in isotonic sucrose apparently possess all of the components necessary to carry out optimal rates of oxidation and phosphorylation. Swollen or disrupted mitochondria, on the other hand, would appear to have lost partially some component essential for the process of oxidative phosphorylation that can be reactivated or replaced by thyroxine. The question of whether thyroxine could control cellular oxidations under physiological conditions by effects analogous to the stimulation of oxygen uptake and P/O ratio in submitochondrial particles would therefore appear to depend on the extent to which the circulating level of the thyroid hormones can influence the intramitochondrial level of thyroxine or of the component replaceable by thyroxine.

No evidence has been found to indicate that the inhibitory actions of thyroxine on phosphorylation that followed some change in the mitochondria or submitochondrial particles during preincubation was ever accompanied by an increase in oxidative rate. This finding, in agreement with that of others,^{2,3} indicated that thyroxine was not acting as a true uncoupling agent, but rather as an inhibitor of phosphorylation. Nevertheless, as pointed out above, any decrease in the efficiency of phosphorylation that occurred *in vivo* would presumably require the cell to increase its rate of respiration to meet its energy

requirements. Clearly it still is not possible to decide whether thyroxine increases the rate of oxygen consumption *in vivo* by lowering the phosphorylation efficiency or by a direct stimulation of the process of oxidative phosphorylation such as we have seen with the submitochondrial particles, or by some other process as yet unknown.

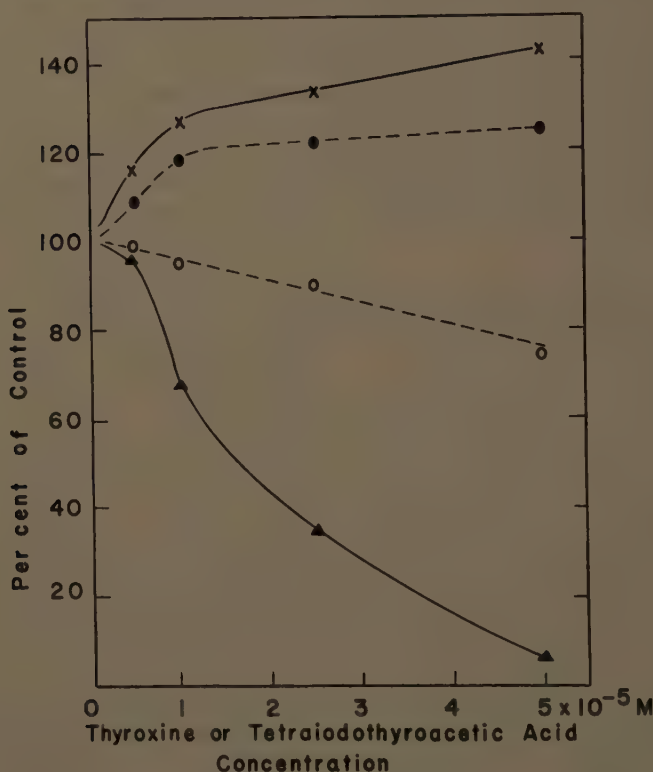


FIGURE 3. The influence of thyroxine and tetraiodothyroacetic acid on rate of oxygen uptake and P/O ratio in submitochondrial particles. Symbols: ●-----●, oxygen uptake in the presence of thyroxine; X—X, P/O ratio in the presence of thyroxine; ○-----○, oxygen uptake in the presence of tetraiodothyroacetic acid; ▲—▲, P/O ratio in the presence of tetraiodothyroacetic acid. Five μ M succinate as substrate; 0.11 mg. N; other conditions as in TABLE 1.

The influence of the acetic acid analogues on oxidative phosphorylation. A possible means of distinguishing between these alternatives has been found in the results of a study that I recently made of the influence of the acetic acid analogues of thyroxine and triiodothyronine on oxidative phosphorylation.¹¹ FIGURE 3 shows a comparison of the influence of thyroxine and tetraiodothyroacetic acid (TETRAC) on rate of oxidation and P/O ratio in submitochondrial particles with succinate as the substrate. The upper 2 curves give the percentage of control values of oxygen uptake and P/O ratio in the presence of varying concentrations of thyroxine, while the lower 2 curves show the per-

centage of control values of oxygen uptake and P/O ratio in the presence of varying concentrations of TETRAC. This data indicates that TETRAC produces effects exactly opposite to those of thyroxine. Concentrations of TETRAC of $1 \times 10^{-6} M$ and above substantially inhibited phosphorylation

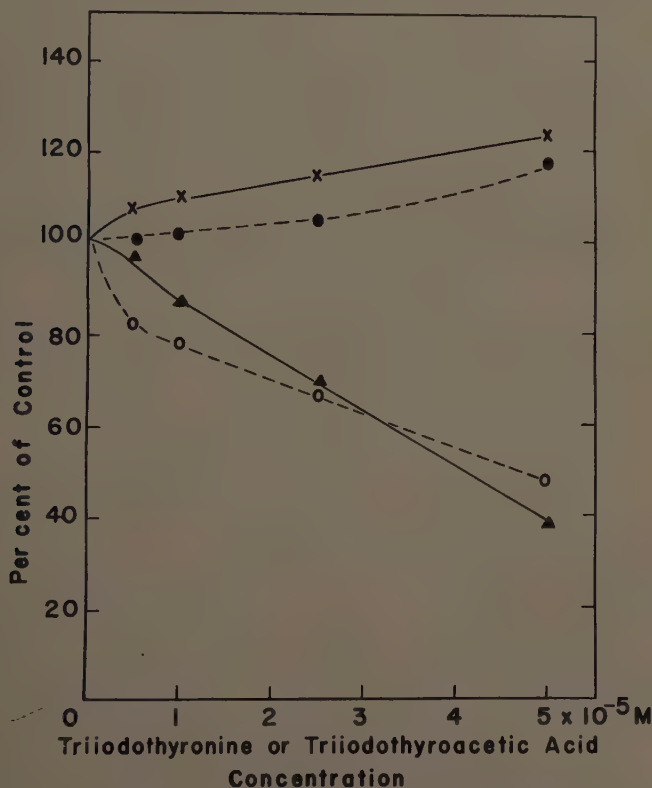


FIGURE 4. The influence of triiodothyronine and triiodothyroacetic acid on rate of oxygen uptake and P/O ratio in submitochondrial particles. Symbols: ●-----●, oxygen uptake in presence of triiodothyronine; ×——×, P/O ratio in presence of triiodothyronine; ○-----○, oxygen uptake in presence of triiodothyroacetic acid; ▲——▲, P/O ratio in presence of triiodothyroacetic acid. Five μM succinate as substrate; 0.10 mg. *N*; other conditions as in TABLE 1.

and to a slight extent inhibited oxygen uptake in the submitochondrial particle preparation.

FIGURE 4 shows a similar comparison of the effects of triiodothyronine and triiodothyroacetic acid (TRIAC) on oxygen uptake and P/O ratio in submitochondrial particles with succinate as the substrate. The upper 2 curves show the effect of triiodothyronine, which is in the same direction as that of thyroxine, but less extensive. The 2 lower curves show the inhibition of phosphorylation and oxygen uptake that were caused by TRIAC. TRIAC was a less potent inhibitor of phosphorylation than TETRAC, but TRIAC

slowed oxidation to a much greater extent than did TETRAC. The inhibitory effects of TETRAC and TRIAC were immediate and did not require any sort of pretreatment of the submitochondrial particles.

TABLE 5 shows a comparison of the effects of thyroxine, TETRAC, triiodothyronine, and TRIAC on intact mitochondria maintained in isotonic sucrose. These incubations were carried out with succinate as the substrate and in the presence of magnesium chloride. It is clear that the acetic acid analogues produced an immediate and very marked inhibition of phosphorylation and some inhibition of rate of oxygen uptake under conditions in which thyroxine and triiodothyronine had no effect. Once again, no preincubation or pretreatment of any kind was used in this experiment.

The data presented above indicate that the acetic acid analogues of thyroxine and triiodothyronine influence the process of oxidative phosphorylation primarily by a direct inhibition of phosphorylation. If these compounds affect

TABLE 5

THE INFLUENCE OF THYROXINE, TRIIODOTHYRONINE, AND THEIR ACETIC ACID ANALOGUES ON OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA

Addition	Oxygen uptake		P/O ratio	
	μ atoms O/min.	Per cent control		Per cent control
None	0.299	—	1.43	—
2.5×10^{-6} M thyroxine	0.295	99	1.46	102
2.5×10^{-6} M tetraiodothyroacetic acid	0.214	72	0.06	4
2.5×10^{-6} M triiodothyronine	0.288	96	1.49	104
2.5×10^{-6} M triiodothyroacetic acid	0.180	60	0.18	13

Conditions: The same as in TABLE 3 except that $10 \mu\text{M}$ MgCl_2 and 0.52 mg. N were present in all incubations. Incubation time 75–120 sec., giving 0.3–0.4 μ atoms of oxygen consumed.

oxidative phosphorylation *in vivo*, there can be little doubt that they would produce the same sort of inhibition of phosphorylation that we have seen with mitochondria and submitochondrial particles.

Conclusion

The action of the acetic acid analogues of thyroxine and triiodothyronine and the inhibition of phosphorylation caused by thyroxine following some change in the mitochondria or submitochondrial particles remain essentially negative effects. The only effect on the process of oxidative phosphorylation that appears to stimulate the rate of oxygen uptake without reducing the efficiency of phosphorylation is the stimulation of oxidation and P/O ratio by thyroxine and triiodothyronine in submitochondrial particles and swollen mitochondria. In order to distinguish between these 2 alternative possibilities for the action of the thyroid hormones on cellular oxidations, more information must be obtained about the relative efficiencies of oxidative processes in the hypothyroid and normal states.

Summary

Studies of the process of oxidative phosphorylation in submitochondrial particles prepared by sonic disintegration of rat liver mitochondria have revealed direct effects of thyroxine and some of its analogues.

Thyroxine and triiodothyronine increased the initial rate of oxidation of succinate on reduced diphosphopyridine nucleotide by the submitochondrial particles. Associated with this increased oxidation rate was an increase in the efficiency of phosphorylation. Tetraiodothyroacetic acid and triiodothyroacetic acid caused an immediate inhibition of phosphorylation, and this effect was associated with a small inhibition of oxygen uptake.

Thyroxine or triiodothyronine produced no effect on intact mitochondria maintained in isotonic sucrose, although their acetic acid analogues caused an immediate inhibition of phosphorylation. Washing intact mitochondria in hypotonic phosphate reduced the rate of oxidation of succinate, and this effect was partially reversed by the addition of thyroxine.

Pretreatment of either mitochondria or submitochondrial particles resulted in a marked inhibition of phosphorylation by thyroxine. This inhibition was reversed by magnesium chloride in intact mitochondria, but not in the particles.

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ENZYME RESPONSES TO THYROID HORMONES

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It has been known for many years that some tissues of animals fed thyroid substance have higher than normal concentrations of respiratory enzymes such as certain dehydrogenases, cytochrome *c* and, possibly, cytochrome oxidase.¹ Before offering these findings as an explanation of the enhanced oxidative metabolism characteristic of hyperthyroidism, it should be recalled that metabolic rates are governed more immediately by work load than by tissue enzyme concentration. Under conditions of rest the rates reflect largely the efficiency with which phosphorylations are coupled to enzymically catalyzed oxidations.² Parenthetically, it should be mentioned that evidence has been presented³⁻⁵ indicating that metabolic control through phosphorylation reactions is indeed less effective in the mitochondria from rats fed thyroid substance than in corresponding preparations from the tissues of control rats. However, the processes by which thyroid hormones bring about such changes are not understood. Actually, the amounts of most dehydrogenases, of cytochromes, and of cytochrome oxidase are far in excess of those required to account for the maximal rate of respiration. Therefore, small increments in these amounts do not seem likely to be the key factor in mediating the thyroid hormone's influence on respiration.

Having thus minimized the presently interpretable significance of the subject of this paper, we shall now describe a very dramatic increase in the amount of a mitochondrial dehydrogenase following thyroid hormone administration.

During an investigation of the interrelation of estrogens and thyroid hormones we attempted to demonstrate the effects of estrogen on the respiration of mitochondria from tissues of rats fed thyroid substance. When experiments with the usual tricarboxylic acid cycle intermediates failed to show *in vitro* the antagonism so easily demonstrated in the intact animal,⁶ we extended our observations to a wider variety of substrates. While the results with estrogens were still unimpressive, we did observe, incidentally, that thyroid feeding brought about a great increase in the rate of oxidation of α -glycerophosphate.⁷

In a system containing 50 μ moles of phosphate buffer, pH 7.4, 15 μ moles of $MgSO_4$, 6 μ moles of adenosine triphosphate (ATP), 60 μ moles of glycerophosphate (40 per cent DL α , 60 per cent β), 0.5 ml. of mitochondrial suspension in 0.25 *M* sucrose, 50 μ moles of glucose, and 1 mg. of a crude yeast hexokinase,* the kidney and liver mitochondria from rats fed 2 per cent desiccated thyroid consumed oxygen 5 times as rapidly as did the mitochondria from control rats. To be more precise, the observed Q_{O_2} (N) (cubic millimeters oxygen consumed per hour per milligram mitochondrial nitrogen) of normal liver mitochondria with glycerophosphate as substrate averaged 57, and that of liver mitochondria from rats fed 2 per cent thyroid for a period of 10 to 20 days was about 250.⁷ We found later that the commercial hexokinase contributed considerable oxidizable substrate to the system and thus introduced an error. TABLE 1 summarizes a series of experiments in which crystalline yeast hexokinase (prepared by an un-

* Type II, Sigma Chemical Company, St. Louis, Mo.

published method of R. A. Darrow and S. P. Colowick) was employed to transfer phosphate from the system to the ultimate acceptor, glucose. The Q_{O_2} (N) values with the substrate-free hexokinase are considerably lower than those reported previously,⁷ and the enhancement by thyroid feeding is now observed to

TABLE 1
RATES OF OXIDATION OF α -GLYCEROPHOSPHATE BY LIVER MITOCHONDRIA OF NORMAL AND THYROID-FED RATS*

Source of liver mitochondria	No. of experiments	Average Q_{O_2} (N)
Normal rats	12	8.0
Rats on diet containing 2% desiccated thyroid	6	180.0

* Measurements were carried out at 30° C.; the reaction components were those described in the text, except that crystalline hexokinase was substituted for the crude commercial preparation.

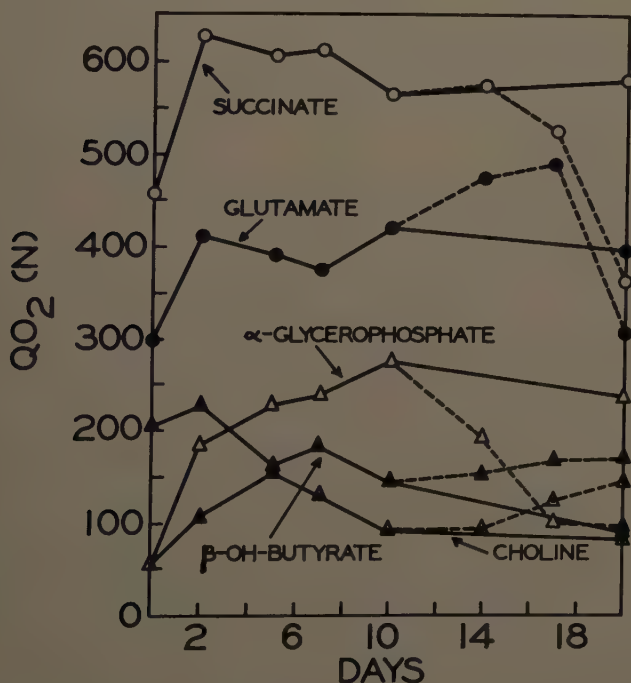


FIGURE 1. Reproduced by permission of *The Journal of Biological Chemistry*.⁷

be more than twentyfold. The experiments summarized below were all done with the commercial hexokinase; the results therefore err in absolute terms, but the differences between normal and thyroid-fed animals are real and readily apparent.

The time course of the response is shown in FIGURE 1. The rate of glycerophosphate

phosphate oxidation reaches a maximum about 10 days after the initiation of thyroid feeding and remains elevated throughout the 20-day experimental period. Glutamate and succinate oxidations increase to about 35 per cent above the normal level within 2 days and then remain at that level. Choline oxidation is enhanced for a few days after initiation of thyroid feeding, but within 10 days returns to nearly the normal level. The oxidation of β -hydroxybutyrate is depressed because this substrate is particularly sensitive to the lower concentration of diphosphopyridine nucleotide (DPN) in mitochondria following thyroid feeding.⁵ Cessation of thyroid feeding causes the rate of glycerophosphate oxidation to decline and, within 10 days, it is near to its original value.

In experiments not summarized in FIGURE 1 it was found that the rate of glycerophosphate oxidation in both liver and kidney mitochondria was signifi-

TABLE 2
EFFECT OF THYROID STATUS ON GLYCEROPHOSPHATE OXIDATION*

Type of rat and treatment	Liver M_w † Q_{O_2} (N)‡	Kidney M_w † Q_{O_2} (N)‡
Normal	57 (10)	87 (6)
1 mg. T_4 , 24 hr.	67	102
1 mg. T_4 , 48 hr.	138	96
0.2 mg. T_3 , 24 hr.	177	152
0.2 mg. T_3 , 48 hr.	187	170
1 mg. TRIAC, 24 hr.	103	110
1 mg. TRIAC, 48 hr.	143	136
Thyroid-fed	276 (5)	236 (5)
Thyroidectomized	36 (5)	14 (3)
0.16 mg. T_3 , 24 hr.	77	
5 mg. T_3 , 24 hr.	106	

* Respiration was measured at 30° C. in the manner described in the text. The values represent averages of duplicate experiments with one rat, except those in parentheses, which indicate a greater number of animals.

† Mitochondria washed twice with 0.25 M sucrose.

‡ Cubic millimeters oxygen consumed per milligram mitochondrial oxygen.

cantly enhanced within 12 hours of initiating thyroid feeding, but not within 6 hours.

When injected subcutaneously into normal rats, triiodothyronine (T_3) elicited a greater response than did thyroxine (T_4) or triiodothyroacetic acid (TRIAC). Thyroidectomy depressed the rate of glycerophosphate oxidation, but the injection of T_3 into thyroidectomized rats enhanced the rate to above normal levels (TABLE 2).

The influence of thyroid substance on the rate of glycerophosphate oxidation is observed as readily with submitochondrial particles as with intact mitochondria (TABLE 3, line 1). This renders unlikely the possibility that differences in permeability are responsible for the more rapid rate of glycerophosphate oxidation by mitochondria from thyroid-fed rats.

The oxidation of glycerophosphate by both normal and hyperthyroid mitochondria is completely inhibited by cyanide (TABLE 3, line 2), indicating that the activity elicited by thyroid feeding is cytochrome-linked as is the normal mitochondrial α -glycerophosphate dehydrogenase. Phenazine methosulfate

has been reported by Ringler and Singer⁹ to accept electrons directly from particulate α -glycerophosphate dehydrogenase. Its use permits direct estimation of the dehydrogenase activity independently of other electron-transferring enzymes that conceivably could be rate-limiting in the mitochondria or particles. As may be seen from the last two lines of TABLE 3, the influence of thyroid substance on rate of glycerophosphate oxidation may be explained entirely by the increased amount of dehydrogenase. A quantitative spectrophotometric assay based on the acceptance by phenazine methosulfate of electrons directly from

TABLE 3
OXIDATION OF α -GLYCEROPHOSPHATE BY SUBMITOCHONDRIAL PARTICLES*

Additions	QO ₂ (N)	
	Normal	Thyroid-fed
Control system	15	123
NaCN, 3 μ moles	2	0
Phenazine methosulfate, 2 mg.*	26	151
CN ⁻ + phenazine methosulfate	23	176

* The system contained 50 μ moles Tris buffer and 10 μ moles phosphate buffer, both at pH 7.4; 10 μ moles KF, 15 μ moles MgSO₄, 6 μ moles ATP. The submitochondrial particles were prepared as described elsewhere.⁸

TABLE 4*
COMPARISON OF α -GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY IN HEPATIC MITOCHONDRIA OF RATS RECEIVING VARIOUS TREATMENTS

Group No.	Treatment	QO ₂ (N) \pm standard error
I	Stock diet (control)	64 \pm 6
II	Fed 2% desiccated thyroid	233 \pm 15
III	Fed 2% desiccated thyroid plus 0.5% DL-ethionine	86 \pm 5
IV	Fed 0.5% DL-ethionine	52 \pm 1
V	Daily intraperitoneal injections of 2 gm./kg. glycerol	66 \pm 8

* From *The Journal of Biological Chemistry*.⁷

the dehydrogenase confirms the twentyfold enhancement (TABLE 1) brought about by thyroid feeding.

The dehydrogenase that becomes more active under the influence of thyroid hormone is specific for L- α -glycerophosphate as its substrate.⁷ It does not attack the related structures, β -glycerophosphate, glycerol, propanediol phosphate, or propanediol. The product of the oxidation, dihydroxyacetone phosphate, accumulates in amounts equivalent to the oxygen consumed. Most tissues contain a second α -glycerophosphate dehydrogenase found in the soluble fraction of the cell; it reacts with DPN. In contrast to the great increase of the mitochondrial α -glycerophosphate dehydrogenase, the amount of soluble dehydrogenase is not at all affected by thyroid feeding.⁷

The data of TABLE 4 demonstrate that ethionine, an analogue of methionine and an inhibitor of protein synthesis, very strongly inhibits the increase of α -

glycerophosphate dehydrogenase. Thus the hormone seems to induce the synthesis of new dehydrogenase protein rather than to activate a latent form of the enzyme.

Thyroid hormone feeding did not influence the activity of brain mitochondrial α -glycerophosphate dehydrogenase, which brings to mind the findings of Gordon and Heming,^{10, cf 11, 12} that only some organs of the body show enhanced respiration following thyroid administration. Brain is one of the tissues reported not to respond in this manner.

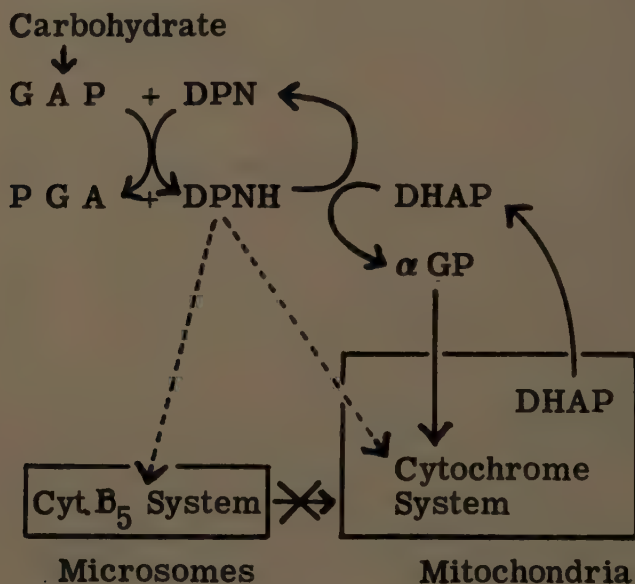


FIGURE 2. Schematic presentation of possible pathways of cellular oxidations. Reduced diphosphopyridine nucleotide (DPNH) generated during the oxidation of glyceraldehyde 3-phosphate (GAP) may diffuse into the mitochondria, react with the cytochrome b_5 system of microsomes, or reduce dihydroxyacetone phosphate (DHAP) to α -glycerophosphate (α GP). The latter may diffuse into mitochondria and be oxidized by the cytochrome-linked dehydrogenase. PGA, phosphoglyceric acid.

Discussion

The question naturally arises whether the enhanced oxidation of so simple a substrate as α -glycerophosphate can have anything to do with the function of the thyroid hormone. While it is far too early to answer the question directly, we may point out some facts about the mitochondrial α -glycerophosphate dehydrogenase that add to its stature as a metabolically important enzyme.

Most dehydrogenases of mammalian cells have been shown to be concentrated within the particulate mitochondrial structures of the cell. Here they are in close proximity to the cytochrome enzymes responsible for the terminal stages of cellular respiration. The rates of oxidations catalyzed by these dehydrogenases are governed by the availability of inorganic phosphate and of phosphate acceptors.² However, there are many dehydrogenases in other parts of

the cell, particularly in the soluble portion of the cytoplasm. These dehydrogenases reduce DPN or triphosphopyridine nucleotides (TPN). The pathway by which the extramitochondrial reduced pyridine nucleotides (DPNH) pass electrons on to the mitochondrial cytochrome system has been the subject of much speculation (FIGURE 2). The reduced pyridine nucleotides may diffuse into the mitochondria and be oxidized directly,¹³ but the rate of such oxidations is low¹⁴ and probably limited by the rate at which the relatively large nucleotides diffuse across the mitochondrial membrane. The microsomal fraction of cells contains cytochrome *b₅*, which is capable of being reduced by pyridine nucleotide and can transfer electrons to cytochrome *c*. However, this system probably does not transfer electrons from soluble pyridine nucleotides to mitochondrial cytochrome in the intact cell, for neither microsomes nor the isolated cytochrome *b₅* can do so *in vitro* (unpublished experiments done in this laboratory).

Another postulated pathway^{15,16} for these oxidations is by way of α -glycerophosphate, as depicted schematically in FIGURE 2. DPNH can reduce dihydroxyacetone phosphate to α -glycerophosphate. The latter may diffuse into the mitochondria, where it is oxidized by the α -glycerophosphate dehydrogenase. The product of this oxidation is dihydroxyacetone phosphate, which is free to diffuse out of the mitochondria and be reduced again. Thus a 3-carbon organic phosphate compound serves as an electron carrier across the mitochondrial membrane. Experimentally we have found that adding dihydroxyacetone phosphate and soluble α -glycerophosphate dehydrogenase to a mitochondrial system will enhance the rate of DPNH oxidation. This finding supports the postulation^{15,16} of the role of mitochondrial α -glycerophosphate dehydrogenase in the oxidation of soluble DPNH. How thyroid hormone influences this pathway is being investigated.

Phillips and Langdon¹⁷ found that thyroid supplements doubled the liver soluble TPNH-cytochrome *c* reductase and postulated that the function of the thyroid hormone is to enhance oxidation of extramitochondrial pyridine nucleotide. The data presented here are in accord with their postulate, but much more work will be needed to determine how close this brings us to an understanding of the effect of thyroid hormone on metabolic rates.

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Part IV. Metabolic Effects of Thyroid Hormones and Their Analogues

COMPARATIVE EFFECTS OF THYROXINE ANALOGUES IN EXPERIMENTAL ANIMALS*

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Introduction

Investigation of the biological effects induced in experimental animals by thyroxinelike compounds was initiated by the early work of Harington.²⁰ Subsequently, renewed interest in thyroxine analogues has developed periodically and culminated in the publication of several review summaries. Following the discovery of triiodothyronine by Gross and Pitt-Rivers,¹⁹ interest again became manifest in thyroxine analogues and their effect on various biological systems, both *in vivo* and *in vitro*. Many of these studies have been covered adequately in recent years by several excellent and comprehensive reviews.^{1,38,49} Therefore, it appears unnecessary to repeat in detail material so thoroughly covered. The present report, instead, includes chiefly those publications and our unreported data that have become available since 1955. Clinical material is not included in this report.

Since no single assay method for thyroxinelike activity measures all possible biological properties of a compound, the principal methods of assay used for such studies have been included in this report. These methods include (1) measurement of metamorphosing ability in the tadpole, (2) inhibition of uptake of I^{131} by the thyroid gland, chiefly in the rat, (3) the prevention of a thiouracil-induced goiter, also mostly in the rat, (4) the effect of compounds on oxygen consumption, primarily in the rat, and (5) prevention of growth of a transplantable, thyroid-stimulating, hormone-secreting, pituitary tumor in the mouse.

Data, both original and those reported by others, showing the relative potency of various analogues compared to thyroxine are summarized in TABLE 1. Since all authors have not used L-thyroxine as a standard, this table has not been corrected for the possible differences that might exist between the L and the DL form of thyroxine. Moreover, in some cases the same author has reported different values for the same compound on different occasions. Other authors apparently have reported the same data in more than one publication. Wherever either of these situations existed, separate reference is made in the table. The compounds listed are those that appear to be of

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major interest and have been studied most frequently. A few other rather unusual chemical compounds have been reported and are omitted from the table. These generally have shown essentially no effect on the end points studied. Some of the compounds listed, as well as many others, have been investigated earlier and are reviewed in detail elsewhere.^{38,49} The interested reader may obtain a great deal of information from these excellent reviews.

*The Effects of Thyroxine Analogues on the
Metamorphosis of the Tadpole*

The data available in the literature would lead the reader to conclude that all anuran tadpoles probably respond to thyroxinelike compounds in a similar manner. However, there have been some suggestions that species differences exist in the quantitative response to a given analogue.⁶⁷ Whether this represents a true difference in sensitivity or only reflects some aspect of the assay method is unsettled. Unfortunately, although an extensive literature is available concerning tadpole metamorphosis, the precise mechanism involved in this phenomenon is still not understood.³⁸ Recent evidence suggests that, in addition to the thyroid gland, steroids may also be involved in regulating the metamorphosis of tadpoles.²³ Certainly, additional studies¹¹ relating to enzymatic changes promise increased knowledge of the mechanisms involved in the process of metamorphosis.

Quantitative data available relating the response of various species of tadpoles maintained in water containing thyroxine or thyroxine analogues frequently show highly variable results. As has been pointed out,³² there are several possible explanations for these discrepant results. In the first place, some compounds are highly insoluble;^{27,32} the material being assayed remains as a suspension in the water in which the tadpoles are maintained. Other compounds added to the tadpole bath in solution may not remain soluble and may precipitate out of the solution. In either case, absorption by the tadpoles may be markedly reduced or nonexistent. Second, the problem of compound stability, either in stock solutions or in the tadpole bath, produces difficulties in evaluating data. The problem of stability for some analogues has recently been discussed in detail.⁵⁴ Finally, the degree of purity existing in the compounds studied can produce fantastic errors in any quantitative calculation. There is little doubt that some of the discrepancies existing in the literature are attributable directly to one or more of these factors. In our previously reported data^{32,33,54} and in some of the material presented below all three of these factors caused concern.

Relative metamorphosing activity of the thyronine series. TABLE 1 lists 39 different analogues (see Selenkow and Asper⁴⁹ for others) and isomers of thyroxine that have been studied in the tadpole. Of all the thyronine compounds tested for metamorphosing activity, the only one reported considerably more active than L-thyroxine is 3,5,3'-triiodo-L-thyronine. Both the DL and the D form of triiodothyronine appear more potent than thyroxine, but are probably less active than L-triiodothyronine. Interestingly enough, replacement in L-triiodothyronine of the hydroxyl group at the 4' position by a methoxy group does not appear to decrease significantly the biological activity. When 2 iodine atoms are in the outer ring with only 1 in the inner (3,3',5'-

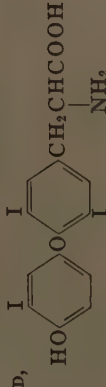
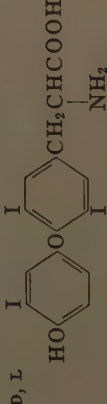
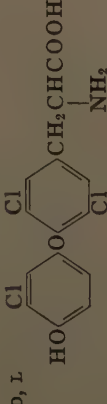
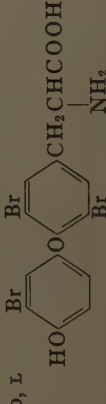
TABLE 1*

Series	Tadpole	I ^{III}	Goiter prevention	B.M.R.	Tumor
Thyronine Series $\begin{array}{c} \text{I} \\ \\ \text{HO} - \text{C}_6\text{H}_3\text{I}_2 - \text{O} - \text{C}_6\text{H}_3\text{I}_2 \\ \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array}$ <p>(3, 5, 3', 5'-tetraiodo-L-thyronine)</p>	100	100	100	100	100
$\begin{array}{c} \text{I} \\ \\ \text{HO} - \text{C}_6\text{H}_3\text{I}_2 - \text{O} - \text{C}_6\text{H}_3\text{I}_2 \\ \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array}$ <p>(3, 5, 3', 5'-tetraiodo-D-thyronine)</p>	50	50 ³³	10 30 ³¹	49	12
$\begin{array}{c} \text{I} \\ \\ \text{HO} - \text{C}_6\text{H}_3\text{I}_2 - \text{O} - \text{C}_6\text{H}_3\text{I}_2 \\ \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array}$ <p>(3, 5, 3', 5'-tetraiodo-D-thyronine)</p>	80-100 55 ⁶² 50 ⁴	40 ³³	72	114	56
$\begin{array}{c} \text{I} \\ \\ \text{CH}_3\text{O} - \text{C}_6\text{H}_3\text{I}_2 - \text{O} - \text{C}_6\text{H}_3\text{I}_2 \\ \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array}$ <p>(O-methyl-D, L-thyronine)</p>	200 ²⁷ 100 ⁵⁷		7 ²⁷ <3 ⁵⁷	33 ²⁷	

D, L 	12 ³² Inact.	<1, 5 ³³	<2 >0.15 ²⁹ >0.15 ³⁵	<1	Inact.
D, L 	4 ³²		5 ²⁹ 0.5 ³⁴ 0.5 ³⁴ 5 ²¹	4 ⁹	
L, 	0 ⁴⁵ 0 ⁴² 0 ⁴		4 ⁹	4 ⁹	
L, 	500 ^{32,42,48} 420 ⁴ 560 ²⁹ 1005 ⁵² 300 ⁴⁶ 500-2000 ⁶⁷ 500 ⁴⁶	300 ³³ 500 ⁷	800 ⁵⁴ 500 ²⁹ 600 ³⁹ 60,19 1000 ⁵⁷ 500-700 ³⁴ 333 ⁹ 500 ⁴⁶	800 ⁶⁴ 200 ¹⁷	100

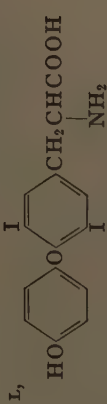
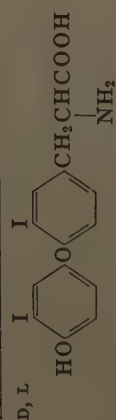
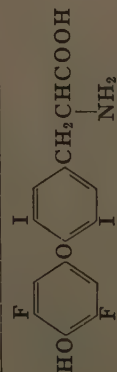
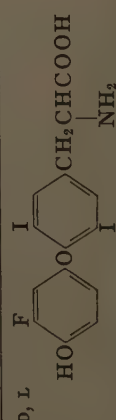
* The superior figures are reference numbers in the bibliography. Where no reference number is given, the data are unpublished observations of the authors. These values are a calculated relative potency value, assuming L-thyroxine as equivalent to 100. The relative potency figures for other authors are all based on a thyroxine standard, but the form used was not always the L-isomer.

TABLE 1 (Continued)

Series	Tadpole	I _{u1}	Goiter prevention	B.M.R.	Tumor
<p>Thyronine Series</p> <p>D, L </p> <p>(3, 5, 3'-triiodo-D, L-thyronine)</p>	300-500 300 ⁵⁷	75	49		
<p>D, L </p> <p>(3, 5, 3'-triiodo-D, L-thyronine)</p>	300-500 500 ⁴⁸ 380 ⁶¹	> 200	429 500 ⁴⁸ 600 ⁸⁹	300	
<p>D, L </p> <p>(3, 5, 3'-trichloro-D, L-thyronine)</p>			129 12 ³⁶		
<p>D, L </p> <p>(3, 5, 3'-tribromo-D, L-thyronine)</p>	56 ⁵²		44 ²⁹ 44 ³⁵ 44 ³⁴		

$ \begin{array}{c} \text{L,} \quad \text{NO}_2 \quad \text{NO}_2 \\ \quad \\ \text{HO} \quad \text{O} \\ \quad \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array} $ <p>(3, 5, 3'-trinitro-L-thyronine)</p>	0 ⁴⁵ 0 ⁴²			250	360	<10
$ \begin{array}{c} \text{L,} \quad \text{I} \quad \text{I} \\ \quad \\ \text{CH}_3\text{O} \quad \text{O} \\ \quad \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array} $ <p>(3, 5, 3'-triiodo-L-thyronine, methyl ether)</p>	> 500					
$ \begin{array}{c} \text{D, L} \quad \text{I} \quad \text{I} \\ \quad \\ \text{HO} \quad \text{O} \\ \quad \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array} $ <p>(3, 3', 5'-triiodo-D, L-thyronine)</p>	2.5 5 ⁴⁵ 5 ⁴²	75 57	<1 ⁵⁴ 5 ⁴⁵ 10 ⁴³	<1 ⁵⁴		Inact.
$ \begin{array}{c} \text{L,} \quad \text{I} \quad \text{I} \\ \quad \\ \text{CH}_3\text{O} \quad \text{O} \\ \quad \\ \text{CH}_2\text{CHCOOC}_2\text{H}_5 \\ \\ \text{NH} \\ \\ \text{CO} \\ \\ \text{CH}_3 \end{array} $ <p>(N'-acetyl-3, 5, 3'-triiodo-L-thyronine, methyl ether, ethyl ester)</p>	25	<5	85	60	25	

TABLE 1 (Continued)

Series	Tadpole	I ¹²¹	Goiter prevention	B.M.R.	Tumor
<p>Thyronine Series</p> <p>L, </p> <p>(3,5-diiodo-L-thyronine)</p>	<p>40³² 0⁴⁵ 0⁴²</p>	<p>37³³</p>	<p>>7⁵⁴ 9 5⁶⁰</p>	<p>11⁵⁴</p>	<p>1</p>
<p>D, L </p> <p>(3,3'-diiodo-D,L-thyronine)</p>	<p>25³² 70⁴⁵ 70⁴²</p>	<p>100³³ 70⁷</p>	<p><1⁵⁴ 12¹⁶ 82⁴⁵ 85⁴³ 100⁴⁴</p>	<p><3⁵⁴ 0¹⁷</p>	<p>Inact.</p>
<p></p> <p>(3,5-difluoro-3',5'-difluorothyronine)</p>			<p>2.8³⁵ <5⁶</p>		
<p>D, L </p> <p>(3,5-diiodo-3'-fluoro-D,L-thyronine)</p>			<p>10.6³⁵ <5⁶</p>		

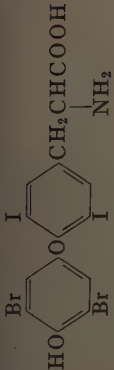
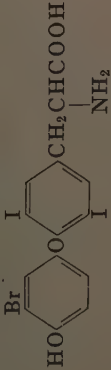
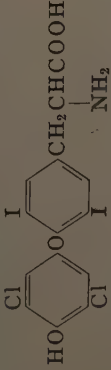
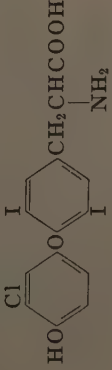
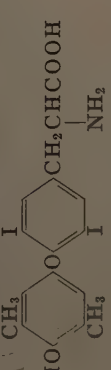
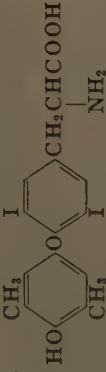
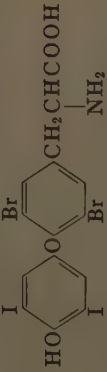
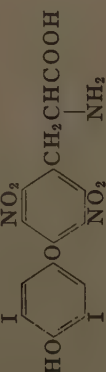
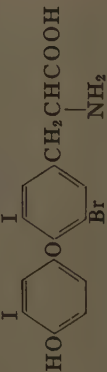
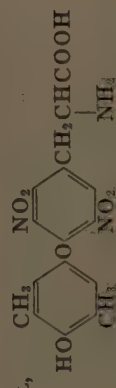
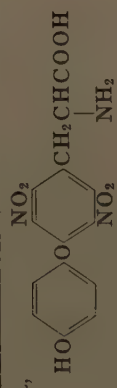
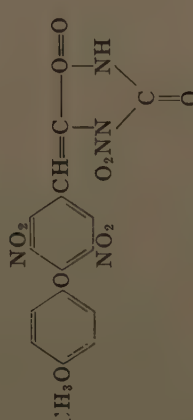
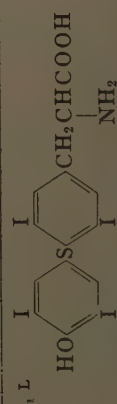
D, L  (3, 5-diiodo-3', 5'-dibromo-D, L-thyronine)					12 ²⁹ 68 ³⁵ 10 ³⁴	49	
D, L  (3, 5-diiodo-3'-bromo-D, L-thyronine)					130 ²⁹ 198 ³⁵ 200 ³⁴		
D, L  (3, 5-diiodo-3', 5'-dichloro-D, L-thyronine)		95 ²⁹			43 ²⁹ 43 ³⁵	III	
D, L  (3, 5-diiodo-3'-chloro-D, L-thyronine)		36 ²⁹			8 ²⁹ 8.2 ³⁵		
L _A  (3, 5-diiodo-3', 5'-dimethyl-L-thyronine)		120 ⁴					

TABLE 1 (Continued)

Series	Tadpole	I ¹³¹	Goiter prevention	B.M.R.	Tumor
<p>Thyronine Series</p> <p>D, L  (3, 5-diiodo-3', 5'-dimethyl-D, L-thyronine)</p>	55 ⁴				
<p>D, L  (3, 5-dibromo-3', 5'-diiodo-D, L-thyronine)</p>			12.5 ³⁶ 12.5 ³⁴	11	
<p>L,  (3, 5-dinitro-3', 5'-diiodo-L-thyronine)</p>			0 ⁶⁰		
<p>D, L  (3, 3'-diiodo-5-bromo-D, L-thyronine)</p>			40 ¹⁶	Act. ¹⁷	

$\begin{array}{c} \text{HO} \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{O} \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array}$ <p>(3-iodo-D, L-thyronine)</p>	0 ⁴⁵ 0 ⁴²				
$\begin{array}{c} \text{HO} \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{O} \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array}$ <p>(3'-iodo-D, L-thyronine)</p>	0 ⁴⁵ 0 ⁴²				
$\begin{array}{c} \text{HO} \\ \\ \text{C}_6\text{H}_3(\text{Br}) \\ \\ \text{O} \\ \\ \text{C}_6\text{H}_4(\text{I}) \\ \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array}$ <p>(3-iodo-3', 5'-dibromo-D, L-thyronine)</p>	Inact.	<3	<2	<2	Inact.
$\begin{array}{c} \text{HO} \\ \\ \text{C}_6\text{H}_3(\text{Br}) \\ \\ \text{O} \\ \\ \text{C}_6\text{H}_3(\text{Br}) \\ \\ \text{CH}_2\text{CHCOOH} \\ \\ \text{NH}_2 \end{array}$ <p>(3, 5-dibromo-3'-iodo-D, L-thyronine)</p>				133 ³⁵ 130 ³⁴	
$\begin{array}{c} \text{CH}_3\text{O} \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{O} \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{CH}=\text{C}-\text{C}=\text{O} \\ \quad \quad \\ \text{N} \quad \quad \text{O} \\ \quad \quad \quad \diagup \quad \diagdown \\ \quad \quad \quad \text{C} \quad \text{C}_6\text{H}_5 \end{array}$ <p>(4-[3'-iodo-4'-(4"-methoxyphenoxy)-benzal]-2-phenyl-5-oxolone)</p>	Inact. ³²	<3			Inact.

TABLE 1 (Continued)

Series	Tadpole	I ₁₂₁	Goiter prevention	B.M.R.	Tumor
Thyronine Series  L, <chem>CC1=CC(=C(C=C1)O)Oc2cc([N+](=O)[O-])cc([N+](=O)[O-])c2CC(N)C(=O)O</chem> (3, 5-dinitro-3', 5'-dimethyl-L-thyronine)	<1 ⁴				
 L, <chem>CC1=CC(=C(C=C1)O)Oc2cc([N+](=O)[O-])cc([N+](=O)[O-])c2CC(N)C(=O)O</chem> (3, 5-dinitro-L-thyronine)			0.25 0.26 0.50		
 (1-nitro-5-[3', 5'-dinitro-4'-(4''-methoxyphenoxy)-benzyl]-hydantoin)	Inact. ³²	<3 ³³	<3	<2	Inact.
 D, L <chem>CC1=CC(=C(C=C1)O)Oc2cc([N+](=O)[O-])cc([N+](=O)[O-])c2CC(N)C(=O)O</chem> (sulfur analogue of thyroxine)	17 ³⁹		0.98 ³⁵ 1.29		

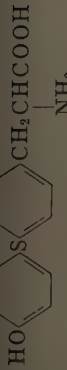
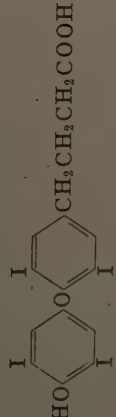
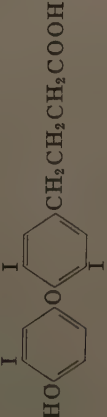
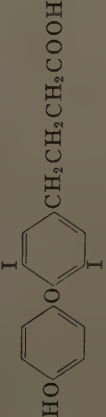
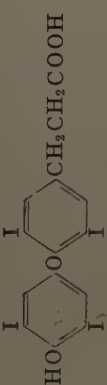
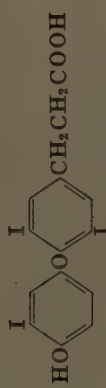
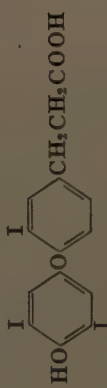
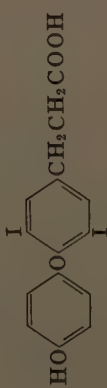
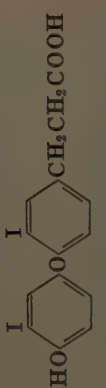
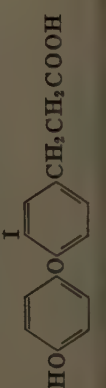
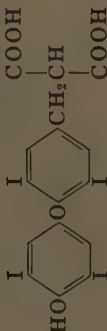
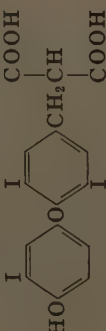
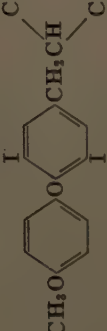
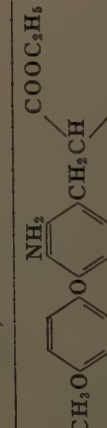

 <p>(sulfur analogue of triiodothyronine)</p>				
<p>Butyric Acid Series</p>  <p>(3, 5, 3', 5'-tetraiodobutyryl acid)</p>	<p>1200³² 10000⁴⁸</p>	<p>30³³</p>	<p>7⁵⁴</p>	<p>5⁵⁴</p>
 <p>(3, 5, 3', 5'-triiodobutyryl acid)</p>	<p>1200³²</p>	<p>75³³</p>	<p>7⁵⁴</p>	<p>Inact.</p>
 <p>(3, 5, 3'-triiodobutyryl acid)</p>	<p>300³²</p>	<p>60³³</p>	<p><2⁵⁴</p>	<p>Inact.</p>
<p>Propionic Acid Series</p>  <p>(3, 5, 3', 5'-tetraiodopropionic acid)</p>	<p>8000³² 12000⁴⁵ 9500⁵⁹ 12000⁴⁷ 12000⁴² 13000⁴ 2100⁵⁷</p>	<p>60³³</p>	<p>14⁵⁴ 75²⁹ 20⁵⁷ 25⁵⁰ 75⁴⁷</p>	<p>5⁵⁴ 10⁵⁷</p>

TABLE 1 (Continued)

Series	Tadpole	µm	Goiter prevention	B.M.R.	Tumor
Propionic Acid Series  (3,5,3'-triiodothyropropionic acid)	30000 ³² 29000 ⁴⁶ 27000 ³⁹ 29000 ⁴² 400-1500 ⁵⁷ 13000 ⁴⁸	60 ³³	28 ⁵⁴ 100 ³⁹ 30 ⁵⁷	10 ⁵⁴ >50 ⁵⁷	10
 (3,3',5'-triiodothyropropionic acid)	10 ³²	50 ³³	<1 ⁵⁴	<1 ⁵⁴	Inact.
 (3,3',5'-triiodothyropropionic acid)	600 ³² 80 ³⁷	16 ³³	<3 ⁵⁴	<2 ⁵⁴ 0 ⁵⁷ 1.9 ³⁷	Inact.
 (3,5-diiodothyropropionic acid)	600 ³²	25 ³³	<1 ⁵⁴	<1 ⁵⁴	Inact.
 (3,3'-diiodothyropropionic acid)	Inact. ³²	25	<2 ⁵⁴	<2 ⁵⁴	Inact.

Chemical Structure	Activity	LD ₅₀	LD ₅₀	LD ₅₀	LD ₅₀
<chem>CC1=CC=C(C=C1)Oc2ccc(CCC(=O)O)cc2</chem> (3-iodothyropropionic acid, methyl ether)	Inact.				
<chem>CC1=CC=C(C=C1)Oc2cc(I)cc(CCC(=O)O)c2I</chem> (3, 5-diiodo-3', 5'-dimethyl thyropropionic acid)	Toxic	25	8	5	Inact.
<chem>Oc1cc(I)cc(CCC(=O)O)c1I</chem> (3, 5-dinitro-3', 5'-diiodothyropropionic acid)			0 ⁶⁰		
<chem>Nc1ccc(Oc2cc([N+](=O)[O-])cc(CCC(=O)O)c2[N+](=O)[O-])cc1</chem> (3, 5-dinitro-3', 5'-diiodothyropropionic acid)	Inact. ³²	Inact. ³³	< 2 ⁵⁴	< 2 ⁵⁴	Inact.
<chem>Nc1ccc(Oc2ccccc2)cc1</chem> (3-aminothyropropionic acid)	Inact.				
<chem>Nc1ccc(Oc2ccc(CCC(=O)O)cc2)cc1</chem> (3-aminothyropropionic acid, methyl ether)	Inact.	Inact. ³³	< 2 ⁵⁴	< 1 ⁵⁴	Inact.
<chem>Nc1ccc(Oc2ccc(CCC(=O)O)cc2)cc1</chem> (3-aminothyropropionic acid, methyl ether)	300 ³² Inact.	Inact. ³³	< 2 ⁵⁴	< 1 ⁵⁴	Inact.

TABLE 1 (Continued)

Series	Tadpole	Im	Goiter prevention	B.M.R.	Tumor
Malonic Acid Series  (3, 5, 3', 5'-tetraiodothyromalonic acid)	< 100				
 (3, 5, 3', 5'-tetraiodothyromalonic acid)	> 200				
 (3, 5, 3'-triiodothyromalonic acid)	Inact. ³²	20	< 3	< 2	Inact.
 (3, 5-diiodothyromethylmalonic acid, methyl ether, diethyl ester)	Inact. ³²	Inact. ³³	< 3 ⁶⁴	< 2 ⁶⁴	Inact.
 (3, 5-diaminothyromethylmalonic acid, methyl ether, diethyl ester)					

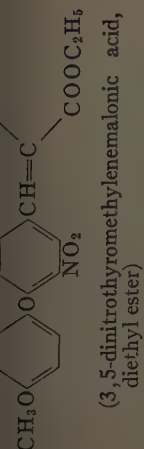
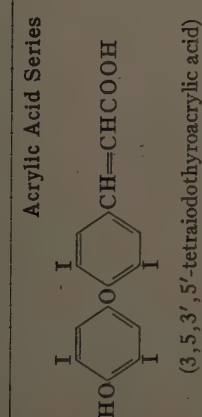
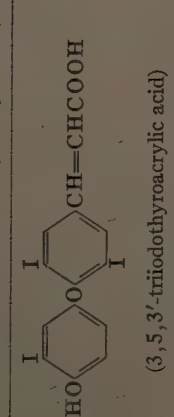
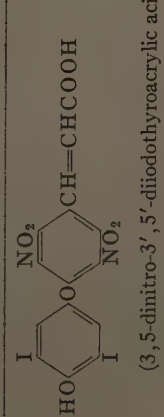
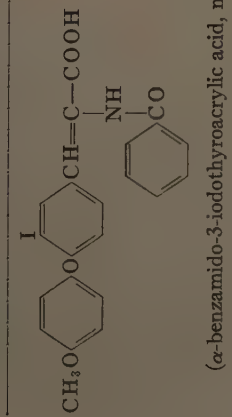
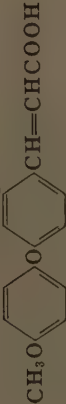

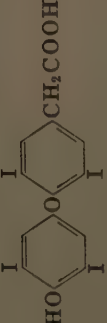
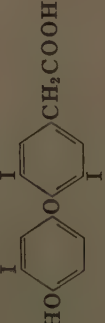
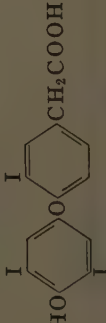
 <p>(3,5-dinitrothymethylenemalononic acid, methyl ether, diethyl ester)</p>					
<p>Acrylic Acid Series</p>  <p>(3,5,3',5'-tetraiodothyroacrylic acid)</p>	<p>24000 2000⁴⁵ 2000⁴² 2900³⁷</p>	<p>50³³</p>	<p>20⁸⁴</p>	<p>13⁸⁴ 25⁵⁰ 2.9³⁷</p>	
 <p>(3,5,3'-triiodothyroacrylic acid)</p>	<p>6500⁴⁵ 6500⁴²</p>				
 <p>(3,5,3'-diiodothyroacrylic acid)</p>	<p>< 1⁴</p>		<p>0⁵⁰</p>		
 <p>(α-benzamido-3-iodothyroacrylic acid, methyl ether)</p>	<p>Inact.</p>	<p>< 2</p>			<p>Inact.</p>

TABLE 1 (Continued)

Series	Tadpole	I ₁₀₁	Goiter prevention	B.M.R.	Tumor
Acrylic Acid Series					
CH_3O  NO_2 (3-nitrohydroacrylic acid, methyl ether)	Inact. ³²	<1.5 ³³	<3 ⁵⁴	<2 ⁵⁴	Inact.
CH_3O  NO_2 (3-nitrohydroacrylic acid, methyl ether, ethyl ester)	1.2 ³²	Inact. ³³	<3 ⁵⁴	<2 ⁵⁴	Inact.
Acetic Acid Series					
 (3,5,3',5'-tetraiodoethoxybenzoic acid)	1000 ³² 1315 ⁵² 1100 ²⁹ 200 ⁴⁸	75 ³³	57 ⁵⁴ 100 ⁵⁷	11 ⁵⁴	50
 (3,5,3',5'-tetraiodoethoxybenzoic acid)	2400 ³² 4280 ⁵² 1900 ²⁹ 12000 ⁴⁸	75 ³³	51 ⁵⁴ 100 ⁵⁷	21 ⁵⁴	50
 (3,5,3',5'-tetraiodoethoxybenzoic acid)	Inact.	15 ³³	<1	<1	Inact.

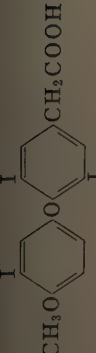
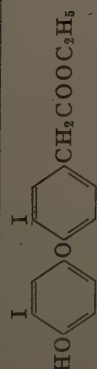
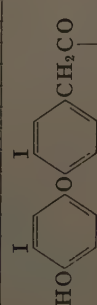
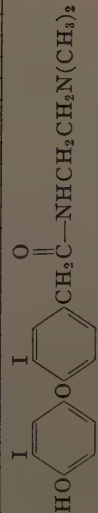
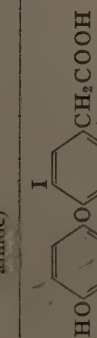
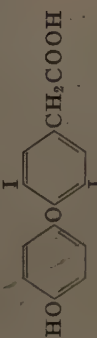
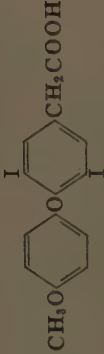
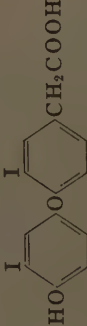
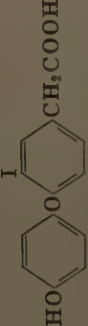
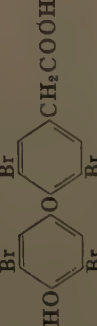
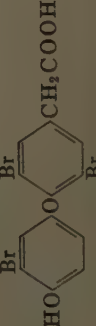
 <p>(3, 5, 3'-triiodothyroacetic acid, methyl ether)</p>	2400	37 ³³	32 ⁶⁴	11 ⁶⁴	7
 <p>(3, 5, 3'-triiodothyroacetic acid, ethyl ester)</p>	2400	7.5	39	10	<1
 <p>(3, 5, 3'-triiodothyroacetamide)</p>	2400	6	5	6	<1
 <p>(3, 5, 3'-triiodothyroacetamide)</p>	1500	7.5	<1	<3	<1
 <p>(3, 5, 3'-triiodothyroacetic acid, β-dimethylaminoethyl amide)</p>	30 ³²	18 ³³	<2 ⁶⁴	<2 ⁶⁴	Inact.
 <p>(3, 5-diiodothyroacetic acid)</p>					

TABLE 1 (Continued)

Series	Tadpole	I ¹²¹	Goiter prevention	B.M.R.	Tumor
Acetic Acid Series  (3,5-diiodothyroacetic acid, methyl ether)	6 ⁵²	50 ³³	<1 ⁵⁴	<3 ⁵⁴	Inact.
 (3,3',5,5'-tetraiodothyroacetic acid)	7	25 ³³	<1 ⁵⁴	<2 ⁵⁴	Inact.
 (3-iodothyroacetic acid)	Inact.	33 ³³	<1 ⁵⁴	<2 ⁵⁴	Inact.
 (3,3',5,5'-tetrabromothyroacetic acid)	21 ⁵²				
 (3,3',5,5'-tetrabromothyroacetic acid)	166 ⁵²				

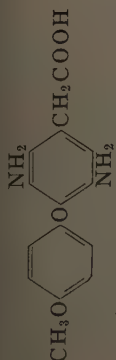
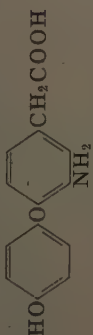
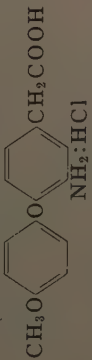
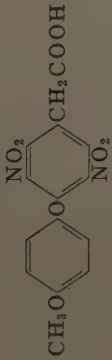
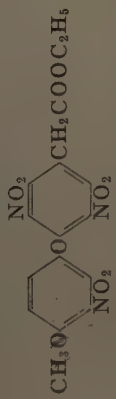
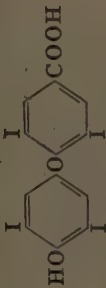
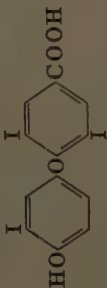
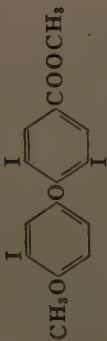
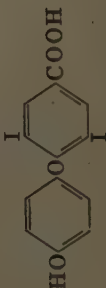
 <p>(3,5-diaminothyroacetic acid, methyl ether)</p>	Inact. ³²	7.5 ³³	< 3 ⁵⁴	< 2 ⁵⁴	Inact.
 <p>(3-aminothyroacetic acid)</p>	Inact.	Inact.			
 <p>(4[4'-methoxyphenoxy]-3-aminobenzyl alcohol hydrochloride)</p>	Inact.	Inact.			
 <p>3,5-dinitrothyroacetic acid, methyl ether)</p>	Inact. ³²	Inact.	< 3 ⁵⁴		Inact.
 <p>(3,5,3'-trinitrothyroacetic acid, methyl ether, ethyl ester)</p>	Inact. ³²	Inact. ³³	< 2 ⁵⁴	< 2 ⁵⁴	Inact.

TABLE 1 (Continued)

Series	Tadpole	I _{an}	Goiter prevention	B.M.R.	Tumor
Formic Acid Series  (3, 5, 3', 5'-tetraiodothyroformic acid)	25 ³² 19 ⁴⁵ 13 ²⁹ 19 ⁴² 0 ⁶⁷ 16 ³⁷	10 ³³	< 2 ⁶⁴ 0 ⁶⁷ 4 ⁶⁰ < 1 ² 0 ¹⁰ < 1 ¹⁹ 0.16 ³⁷	< 2 ⁵⁴ < 1 ²	Inact.
 (3, 5, 3'-triiodothyroformic acid)	200 ³² 120 ⁴⁵ 155 ²⁹ 120 ⁴² 10 ⁶⁷	15 ³³	0 ⁶⁷ < 1 ⁹	0 ⁶⁷	
 (3, 5, 3'-triiodothyroformic acid, methyl ether, methyl ester)	75	15 ³³			Inact.
 (3, 5-diiodothyroformic acid)	Inact. ³² 0 ⁶⁷	15			Inact.

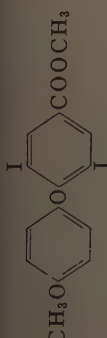
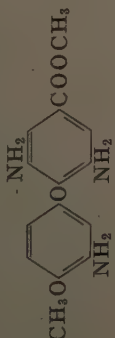
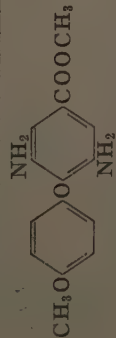
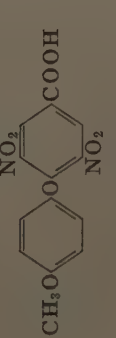
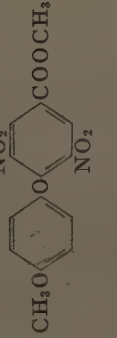
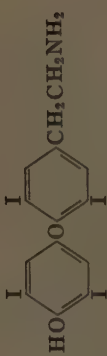
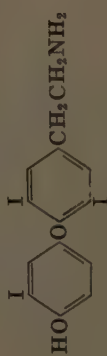
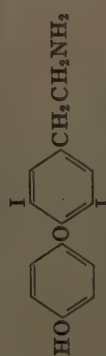
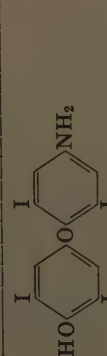
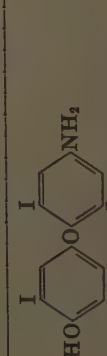
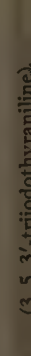
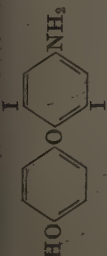

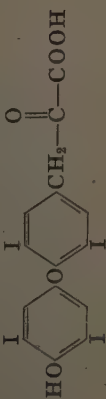
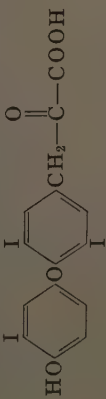
 <p>(3,5-diiodothyroformic acid, methyl ether, methyl ester)</p>	Inact. ³²	Inact. ³³ 15	<3 ⁵⁴	<2 ⁵⁴	Inact.
 <p>(3,5,3'-tri-aminothyroformic acid, methyl ether, methyl ester)</p>	Toxic ³²	Inact. ³³	<3 ⁵⁴	<2 ⁵⁴	Inact.
 <p>(3,5-diaminothyroformic acid, methyl ether, methyl ester)</p>	Toxic ³²	Inact. ³³	<3 ⁵⁴	<2 ⁵⁴	Inact.
 <p>(3,5-dinitrothyroformic acid, methyl ether)</p>	Inact. ³²	Inact. ³³	<3 ⁵⁴	<2 ⁵⁴	Inact.
 <p>(3,5-dinitrothyroformic acid, methyl ether, methyl ester)</p>	Inact. ³²	Inact. ³³	<3 ⁵⁴	<2 ⁵⁴	Inact.

TABLE 1 (Continued)

Series	Tadpole	I _{II}	Goiter prevention	B.M.R.	Tumor
Other Compounds					
<div> (3, 5, 3', 5'-tetraiodothyroxamine)</div>	900 ⁵⁷ 2 ⁴⁸		1 ⁵⁷	10 ⁵⁷	
<div> (3, 5, 3', 5'-tetraiodothyroxamine)</div>	3600 ⁵⁷		11 ⁵⁷	10 ⁵⁷	
<div> (3, 5, 3'-triiodothyronamine)</div>	10 ⁵⁷		1 ⁵⁷		
<div> (3, 5-diiodothyronamine)</div>	10 ⁵⁷ 450 ³⁷		< 1 ⁵⁷	0 ⁵⁷	
<div> (3, 5, 3', 5'-tetraiodothyraniline)</div>	8 ⁵⁷		1 ⁵⁷	0 ⁵⁷	
<div> (3, 5, 3'-triiodothyraniline)</div>					

 <p>(3,5-diiodothyraniline)</p>	10 ⁵⁷ 16 ³⁷		<1 ⁵⁷		
 <p>(3,5,3',5'-tetramethylaniline)</p>	<1 ⁴				
 <p>(3,5,3',5'-tetraiodothyropyruvic acid)</p>	30 ⁴² 30 ⁴⁵		75 ⁴⁵		
 <p>(3,5,3',5'-triiodothyropyruvic acid)</p>	100 ⁴² 100 ⁴⁵		100 ⁴⁵		
<p>(sodium iodide) (3-monoiodo-1-tyrosine) (3,5-diiodo-1-tyrosine) (3,5,3'-diiodo-1-tyrosine) (n-butyl-3,5-diiodo-4-hydroxy-benzoate)</p>	Inact. Inact. 1 0.1 ⁴⁸ Inact. Inact.	20-40 ³¹ 16 15 200 ²⁸			Inact. Inact. Inact. Inact. Inact.

triiodo-DL-thyronine), the metamorphosing activity is reduced to almost zero.^{32,42,45}

There has been some question in the past concerning the biological potency of the racemic and D form of thyroxine. In our assays we have found both forms to vary depending on the samples investigated. Some DL preparations show as much activity as L-thyroxine, while others appear to be less active. Although D-thyroxine has also appeared variable, most of our evidence suggests that this form has about one half the activity of L-thyroxine.

There seems little doubt that the replacement of iodine by 4 atoms of bromine or chlorine reduces the metamorphosing potency of the molecule to very low values. Tribromthyronine, however, apparently is more active than the tetrabrominated thyronine.⁵² Whether the same holds for trichlorothyronine is not certain. It has been reported that the trihalogenated compounds are more active than the tetrahalogenated, with only one possible exception;⁵² namely, 3,5-diiodo-3',5'-dichlorothyronine, which is 2 to 3 times more active than 3,5-diiodo-3'-chlorothyronine.²⁹

All thyronines with iodines in the 3,5 position seem to show evidence of some biological activity on tadpoles; this is also true for 3,3'-diiodothyronine. Monoiodinated compounds have shown little or no biological activity.

Relative metamorphosing activity of the thyrobutyric acid series. There have been few reports on compounds of the butyric acid series and metamorphosis. Our own study³² has been done with compounds that were unquestionably impure. The nature of the contaminants, unfortunately, was not determined. With the limitation of purity in mind, however, it appears that both the tetra- and triiodinated forms have considerably more activity on metamorphosis than L-thyroxine. The 3,5-diiodinated form also appears more active than thyroxine, but much less than the other 2 butyric compounds. Obviously, the studies must be repeated with chromatographically pure compounds before these observations are meaningful.

Relative metamorphosing activity of the thyropropionic acid series. Twelve structurally related compounds of this series have been investigated for their effect on tadpoles. Seven have shown little or essentially no effect in producing metamorphosis; others have been found to be highly potent. Biological activity has been reported to range from a trace to the most active compound yet reported, 3,5,3'-triiodothyropropionic acid, which is approximately 300 times more potent than L-thyroxine.^{29,32,42,45} Moreover, the tetraiodinated form is also highly active,^{4,29,32,42,45,47} and both the 3,5- and 3,3'-diiodinated propionic acid compounds have been reported more active than thyroxine.³² In contrast to the highly active 3,5,3' compound, the 3,3',5' form is extremely low in potency.³² In an earlier report we found the non-halogen-containing compound 3-aminothyropropionic acid, methyl ether, ethyl ester capable of inducing metamorphosis.³² Chemical analysis of this compound failed to show evidence of a halogen. Subsequent studies using a different sample of the same compound failed to substantiate the original observation.

Relative metamorphosing activity of the thyroacetic acid series. Of the 18 thyroacetic acid compounds studied for metamorphosing effect, only the tetraiodinated, the triiodinated, and the tribrominated compounds have been re-

ported to be more active than L-thyroxine. The only exception to this is 3,3',5'-triiodothyroacetic acid, which is inactive. It has been indicated above that, in general, the triiodinated analogues are more active than the tetraiodinated forms. This is also the case with the triiodinated thyroacetic acid (3,5,3'), which has been reported more active than the tetraiodinated form.^{29, 32, 52} The activity of the former compound does not appear changed substantially by the formation of a methyl ether, an ethyl ester, or an acetamide. It is also of interest that the β -dimethylaminoethyl amide of 3,5,3'-triiodothyroacetic acid is at least one half as potent as 3,5,3'-triiodothyroacetic acid. Removal of iodines with only the 3,5 or 3,3' positions intact reduces biological activity to much less than that shown by thyroxine. Formation of a methyl ether in the 3,5 compound seems to result in a further decrease of biological activity.

Relative metamorphosing activity of the thyroformic acid series. Only 3 of the 9 formic acid compounds have shown any biological effect on metamorphosis. The tetraiodinated form has uniformly been found less active than thyroxine and, in general, the 3,5,3' compound more active than thyroxine.^{29, 32, 42, 45, 47} We have found 3,5,3'-triiodothyroformic acid, methyl ether, methyl ester, to be approximately 75 per cent as active as L-thyroxine.

Relative metamorphosing activity of other compounds. Because of their structural similarity to the propionic acid compounds, it is not surprising that the tetra- and triiodinated acrylic compounds are more active than thyroxine in producing metamorphosis. Whether these 2 compounds are more active than their propionic acid counterparts is doubtful. There is, however, little question but that the triiodinated acrylic compound is more active than the tetraiodinated one.^{42, 45} With the limited amount of information available, it appears that the tetra- and triiodinated malonic acid compounds are active, although quantitative values are lacking. Tetraiodothyroxamine and triiodothyroxamine apparently are quite active in the tadpole. The pyruvic acid analogues have been reported capable of inducing metamorphosis, but are no more active than L-thyroxine.

The Effect of Thyroxine Analogues on the Suppression of I^{131} Uptake by the Thyroid

Interpretations of single uptake studies are difficult to evaluate because of the complex nature of thyroid gland function.¹ Single determinations fail to measure all of the essential aspects of thyroid activity relating to trapping, binding, and secretion rates, which may vary widely under different conditions. As our understanding of thyroidal iodine metabolism increases, the shortcomings of the iodine suppression technique become more apparent. However, even considering these limitations, useful information may be obtained using this method. Certainly any major differences in I^{131} uptake observed following administration of compounds to experimental animals could point the way to studies that would ultimately lead to a better understanding of the mechanism of action involved.

A substantial number of thyroxine analogues has been studied, using the uptake of I^{131} as an indication of biological effectiveness in experimental animals.^{3, 7, 28, 30, 31, 33} On this basis, only 3,5,3'-triiodo-L-thyronine has consist-

ently been observed to be more effective than L-thyroxine in preventing the uptake of I^{131} by the thyroid gland. Most of these determinations have used 24 hours following injection of the isotope as the time at which I^{131} measurements were made. The possibility that some compounds may show differences at times other than that generally used has not been systematically investigated.

It appears unlikely that the number of iodine atoms in the analogue molecule is the major factor involved in the blocking of thyroidal I^{131} collection. This is apparent, since D-thyroxine appears to be about one half as effective as the L-isomer, and DL-thyroxine is probably no more active than the D-form. Moreover, 3,5,3'-triiodothyronine is considerably more effective than L-thyroxine, while 3,3',5'-triiodothyronine is less effective than thyroxine.

Apparently all analogues that contain iodine are capable of depressing I^{131} uptake when given in adequate doses. This is not surprising, since the same effect is observed with iodinated tyrosines and sodium or potassium iodide. Therefore, caution should be exercised in interpreting results of this type, since the rate of deiodination of most of these analogues is not known.

The Effect of Thyroxine Analogues on Prevention of a Thiouracil-Induced Goiter

A comparison of the analogues using the rat as an experimental animal has shown only 3,5,3'-triiodothyronine to be markedly more effective than L-thyroxine in preventing the development of a thiouracil-induced goiter. There is some question concerning the relative potency, however.^{9,29,34,39,50,54,57} In addition, the sulfur analogue of triiodothyronine appears to be slightly more effective than thyroxine. There are reports^{29,34,45} that both 3,5-dibromo-3'-iodothyronine and 3,5-diiodo-3'-bromothyronine are somewhat more active than thyroxine. In the case of these 2 compounds this seems somewhat surprising, since 3,5-diiodothyronine appears to have little if any ability to prevent the development of a thiouracil goiter,^{50,54} and there seems to be a wide difference of opinion regarding the relative potency of 3,3'-diiodothyronine.^{16,43-45,54} Moreover, tetra- and tribrominated compounds are less active than L-thyroxine.

The triiodinated thyroacetic acid (TRIAC), and the tetraiodinated compound (TETRAC), both show approximately one half the activity of L-thyroxine.⁵⁴ This is in contrast to the results observed with the tadpole method of assay, where TRIAC was considerably more active than TETRAC. Substitution in the triiodinated compound of a methoxy group in place of the 4' hydroxyl results in further reduction of biological activity.⁵⁴

The presence of 2 iodines on the outer ring and only 1 on the inner (3,3',5'-triiodothyroacetic acid), as shown for other compounds, results in an analogue ineffective in blocking the formation of a goiter. There appears to be a difference of opinion regarding the relative potency of the tetra- and triiodinated propionic acid analogues.^{29,47,50,54,57} In all cases, however, they have been reported to be no more active than thyroxine.

An increase in the number of carbons in the side chain uniformly results in decreased biological activity.⁵⁴ For example, the acetic analogue (with 2 carbons) is more active than the acrylic acid (3 carbons) or the propionic acid (3 carbons). Adding a fourth carbon to make the butyric compound results in

even further reduction in activity. In this latter case, as pointed out elsewhere,³² the question of compound purity casts some doubt on the validity of this comparison. Reduction of the side chain, however, to 1 carbon (formic acid) results in a complete loss of biological activity.

The Effect of Thyroxine Analogues on Oxygen Consumption

It is reasonable to assume that all thyroxine analogues affect oxygen consumption in rats through the same mechanism, but the doses of various compounds required to produce the same response vary considerably.⁵⁴ Of the analogues examined, only 3,5,3'-triiodothyronine has consistently been reported to increase oxygen consumption more effectively than thyroxine.⁵⁴ Most of the other thyronines are less than 25 per cent as effective as thyroxine in elevating oxygen consumption. Those that show any substantial activity are compounds containing iodines in the 3,5,3' positions.

Where comparative studies are available,⁵⁴ the 3,5,3'-triiodothyroacetic acid analogue has been found somewhat more potent in increasing oxygen consumption than the tetraiodinated form. Whether such differences are real or reflect chemical decomposition of the latter compound is at present not clear. Certainly, as may be seen in TABLE 1, there is little doubt that most of the other analogues studied show little if any effect on oxygen consumption in experimental animals. There is, however, some question regarding the relative effectiveness of 3,5,3'-triiodothyropropionic acid.^{54,57}

The Effects of Thyroxine Analogues on the Suppression of Growth of a Transplantable TSH-Secreting Pituitary Tumor

At the present time few studies have used this method to determine the biological potency of thyroxine analogues. The method developed from the observations of Gorbman¹⁸ and later by Furth and Burnett,¹⁴ that administration of radioiodine to mice resulted in destruction of the thyroid gland and ultimately resulted in the development in these animals of pituitary tumors. These tumors could be transplanted into other radiothyroidectomized mice and, after many transplantations, into normal hosts. It was subsequently reported¹⁵ that the administration of thyroxine and thyroglobulin to such tumor-bearing mice inhibited the growth of the tumor transplants. In our studies we have utilized this technique and have investigated the effects of some 60 analogues and isomers of thyroxine on this type of tumor. We have attempted to determine whether any substantial difference existed on a quantitative basis between the compounds studied. Our method²⁴ for evaluating tumor effectiveness, briefly, is to transplant tumor tissue into mice and, when growth of the implant reaches adequate size, administer the various compounds daily to the animals. After 7 weeks of treatment experimental tumors are compared with controls and the degree of inhibition of growth produced by the analogues calculated. It was found that for active compounds the depression of tumor growth is roughly proportional to the dose administered. Repeated assay of L-thyroxine indicated that about 0.26 μ g. were needed to induce a 50 per cent depression in the size of the tumor. This compound was given an arbitrary value of 100, and comparison was made with other compounds. On this basis

D-thyroxine appears to be approximately 10 per cent as effective as L-thyroxine, and DL-thyroxine was found to contain about one half the activity of the L-form.

Of the compounds investigated, most had little or no effect in preventing growth of the tumors. As may be seen from TABLE 1, only 3,5,3'-triiodo-L-thyronine was as effective as L-thyroxine. The other active analogues ranged from tetraiodothyroacetic acid and triiodothyroacetic acid (both one half as active as thyroxine) to several, including the tetra- and triiodinated propionic acid analogues which ranged between one tenth and one twentieth as active as thyroxine.

Examination of TABLE 1 suggests that this method of determining biological activity probably measures the same effect as determination of potency based on the prevention of a thiouracil-induced goiter.

Discussion

The metamorphosing potency of thyroid preparations was considered for many years to be parallel with the ability of these materials to elevate oxygen consumption in higher animals. This observation does not hold for thyroxine analogues, since many effective in the tadpole are almost incapable of altering the metabolic rate of higher animals. Because many compounds effective on metamorphosis have no effect on oxygen consumption, it has been suggested that the tadpole test is nonspecific.³⁷ Nevertheless, amphibia seldom respond to compounds that are totally inactive when tested in mammals. Moreover, there is no question that the tadpole test is useful in preliminary screening of compounds for potential biological activity. It may be that many active compounds would be considered specific for the tadpole if the basic understanding of the metamorphosing process were known.

Recent studies^{36, 53, 55} indicate that 2 different species of animals may respond differently to the same hormone. For example, it is generally agreed that 3,5,3'-triiodothyronine is more effective in the rat in preventing a thiouracil-induced goiter than is thyroxine (see TABLE 1). However, this does not seem to be the case in chickens; in fact, the reverse is true.³⁶ There is evidence indicating that this difference represents, in part, a fundamental difference in the mode of peripheral hormone transport, since the normal thyroxine-binding protein of the higher mammals is missing in chicken and duck blood.⁵⁵ As pointed out,⁵³ however, this would at most be only a partial explanation of the difference in response between the 2 species. It may be of interest that, despite this difference, birds show the same relative response seen in higher experimental animals when 3,5,3'-triiodothyronine is compared with 3,3',5'-triiodothyronine. In all animals thus far studied the latter compound is relatively ineffective on oxygen consumption and goiter prevention.

Although there is uniform agreement that 3,3',5'-iodinated compounds are less potent than the 3,5,3' compounds, the explanation for this difference is not entirely clear. The difference may be partly a reflection of the rate of deiodination of the 2 compounds.³³ What role the binding of some of these compounds to the thyroxine-binding protein plays in the relative effectiveness of the compounds has not been clarified. The report that rat liver contains enzymes capable of metabolizing thyroxine to compounds containing iodine in the 3,5 but not the 3',5' positions supports the concept that compounds with

iodine in the outer ring would be biologically unstable.⁴⁰ In contrast to this, however, are the observations⁵⁹ that I^{131} -labeled 3,5,3'-triiodothyroformic acid and 3,5,3',5'-tetraiodothyroformic acid are deiodinated to a lesser extent in the intact rat than is either thyroxine or triiodothyronine.

There is little doubt that deiodination rates and liberation of iodine will directly influence the thyroidal accumulation of the halogen. As pointed out above, this makes interpretation of single thyroidal I^{131} studies difficult to evaluate. Where such types of study have been done the conclusion has generally been reached that iodine liberated from the compounds probably acts locally on the thyroid.^{3,28,31,33} Thyroid hormone iodine, however, has a direct influence on the anterior pituitary gland. This effect has been demonstrated by a host of studies and is generally accepted as proved. Resolution of the question of whether thyroxine or any of its analogues has a direct effect on the thyroid gland, thus altering thyroid function, is a complex problem and is not completely clarified.

One point that demands further investigation concerns the relative effectiveness of a given compound with respect to the route of administration. There have been numerous indications in the literature that the biological potency of a compound varies depending on the route of administration. This might help explain, in part, the latency period observed with some compounds.^{8,56} We have found, as have others,^{17,38} that thyroxine is considerably less effective orally than when given to animals by the subcutaneous route. On the other hand, both 3,5,3'-triiodothyronine and 3,5,3'-triiodothyropropionic acid do not show this difference. This raises the interesting possibility that some compounds, presently considered inactive, might show significant activity when administered by different pathways.

On the basis of presently known thyroid hormones it appears that the greatest biological effectiveness in mammals⁴¹ is obtained with thyronines containing iodine in the 3,5,3' positions. Methylation at the 4' position produces a compound containing less biological activity. The removal of iodine at 5 and substitution at the 5' position results in almost complete loss of activity. Although there is some discrepancy concerning compounds containing iodine only in 3,5 or 3,3' positions, such preparations may be no more active than 3,3',5' iodinated thyronines. Monoiodinated compounds are uniformly inactive in mammals.

An intact alanine side chain does not seem absolutely essential for biological activity in mammals, but there may be some relation between side-chain length and the activity a compound shows.⁵⁰ As indicated above, this may not hold for amphibia.

It is of interest that iodine can be replaced by other halogens, with retention of activity.^{4,13,16,21,35,38,44,52} With halogen substitution, however, activity decreases in descending order from iodine to bromine to chlorine. Exactly what this signifies from a chemical standpoint is not entirely certain, although theoretical correlations between chemical structure and activity have been postulated.^{5,12,13,22,24,38,58}

One point worthy of comment concerns the possibility that the various effects of thyroxine can be separated by using thyroxine analogues. Recently much effort has been expended in this type of study, and data suggesting this possi-

bility have been reported.^{2,9,13,41a} There is little doubt that biologically active compounds (in experimental mammals) in sufficient doses will exert the same qualitative effect on a given response. However, the quantity of compound necessary to elicit the same magnitude of effect in this response may be vastly different. It also is true that, at a given dose, one response may be marked, while another type of response may be absent. On the basis of this it may be concluded that, at a given dose level, the separation of 2 different effects has been obtained. However, this type of observation is fairly common in biology; for example, with estrogens it probably represents a difference in threshold dosage, since increasing the amount of material given will frequently cause a change elsewhere. True separation of responses would require a molecule whose administration produced only 1 biological effect regardless of the dose given. Whether the present concept of biological separation using analogues is only a matter of semantics remains for the future to determine.

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Addendum

After this manuscript was completed, the authors received additional information, presently unpublished, from Rosalind Pitt-Rivers, National Institute of Medical Research, London, England, relating to recent studies of new compounds measured by the prevention of a thiouracil-induced goiter in rats. Pitt-Rivers has given us permission to quote the following information: D,L-thyroxine 100; tetraiodothyrolactic acid 16; 3,5,3'-triiodothyrolactic acid 25; 3,5-diiodothyrolactic acid <2.5; 3,5,3',5'-tetraiodothyropyruvic acid 13; 3,5,3'-triiodothyropyruvic acid 53. With L-thyroxine 100; 3,3'-dichloro-5,5'-diiodo-L-thyronine 16.4; 3,3'-dibromo-5,5'-diiodo-L-thyronine 17.8.

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THYROXINE ANTAGONISM BY PARTIALLY IODINATED THYRONINES AND ANALOGUES*

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A decade ago several laboratories were concerned with inhibition of thyroxine (T_4) shown by a variety of synthetic compounds vaguely related to the hormone. These were mostly derivatives of 3,5-diiodo-4-hydroxybenzoic or iodo-phenoxyacetic acids. In general, high dosages were required,¹ involving molar ratios of antagonist to T_4 of from 250:1 to 2000:1.

With the recent availability of synthetic 3-iodothyronine (T_1), 3,3'-diiodothyronine (3,3'- T_2), and 3,3',5'-triiodothyronine (3,3',5'- T_3), as well as their acetic and propionic acid analogues, various laboratories² including ours have found them to possess essentially no metabolism-stimulating properties (unpublished observations). This aroused our interest in the possibility that these unusual iodothyronines would interfere with the actions of T_4 and 3,5,3'-triiodothyronine (TRIT, T_3 , 3,5,3'- T_3).³

Whole-animal Metabolism

As the most direct approach to the study of T_4 antagonism, we evaluated these compounds in thyroidectomized rats whose basal metabolic rates (B.M.R.) were maintained by daily injection of 10 to 20 μ g. T_4 /kg. body weight. This, of course, means that the antagonistic effects were exerted on exogenous hormone.

FIGURE 1 shows the series of changes in B.M.R. resulting from combining 3,3',5'- T_3 and T_4 in a group of thyroidectomized rats. Horizontal line 1 represents the 7-day average of 89 ml. O_2 /100 gm. body weight/hour. From this level, the daily injection of 6 and then 10 μ g. T_4 /kg. body weight raised the oxygen consumption to a plateau of 109 ml. (line 2). Then 2 mg. of 3,3',5'- T_3 was superimposed, line 3 showing a prompt fall in metabolic rate to 86, slightly below the original B.M.R. When the 3,3',5'- T_3 was withdrawn while T_4 was continued, the B.M.R. returned to the previous value of 109 on replacement therapy, represented by line 4. Repetition of an 8-day period on 3,3',5'- T_3 again lowered the metabolic rate to approximately the pre- T_4 level, and cessation resulted in a rise. To complete the cycle, T_4 was stopped and, within 3 weeks, the B.M.R. had returned completely to the thyroidectomy value.

A similar action is shown in FIGURE 2, which also emphasizes the competitive nature of the antithyroxine action of 3,3',5'- T_3 , on a molar basis. When the animals were stabilized on 20 μ g. T_4 /kg./day, addition of 3,3',5'- T_3 at 50:1 decreased the B.M.R. 27 per cent of the previous elevation. When 3,3',5'- T_3 was increased to 100:1, the B.M.R. fell to somewhat below the thyroidectomy level. At this point the 3,3',5'- T_3 dose was held constant and the T_4 increased

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5 times, so that the ratio was re-established at 20:1. Within a few days the metabolic rate rose to its previous level of T_4 . Although 3,3',5'- T_3 was then in turn increased to restore the ratio to 100:1, the B.M.R. fell slowly, perhaps because the total dose levels were now extremely high and insufficient time was

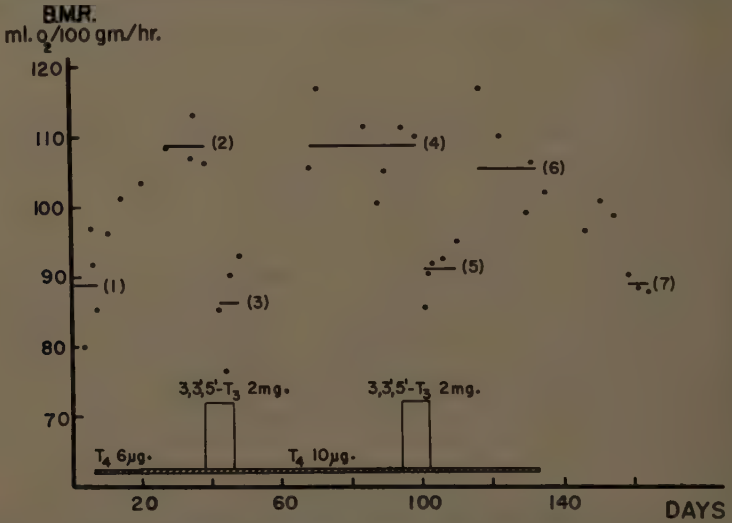


FIGURE 1. B.M.R. of thyroidectomized rats as altered by T_4 and 3,3',5'- T_3 at dosage combinations shown. Numbered horizontal lines represent metabolic rates averaged over the time periods shown on the abscissa. Detailed explanation is in text. Reproduced by permission of *Endocrinology*.³

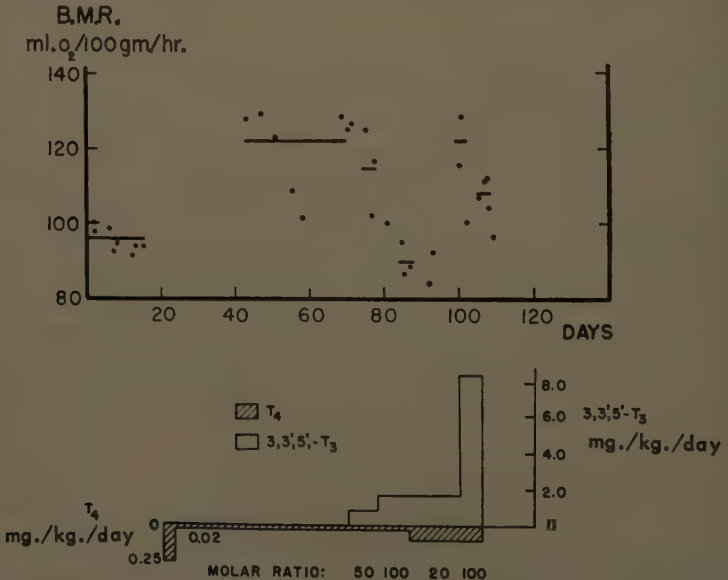


FIGURE 2. Effect on thyroidectomized rat B.M.R. of T_4 and 3,3',5'- T_3 at varying dosages as shown in lower portion of figure. When 3,3',5'- T_3 was started, the resultant T_3 : T_4 molar ratio is shown below the bars representing dosages. Adapted by permission of the *American Journal of Physiology*.¹³

allowed for complete readjustment. Up to this point the ease of reversibility of the T_4 -inhibiting effect suggests competition with T_4 for similar biochemically active sites of action.

FIGURE 3 illustrates the procedure used to compute the extent of T_4 inhibition brought about by the various substances evaluated. Horizontal line (1) represents the average B.M.R. of 82.9 ml. O_2 /100 gm. body weight/hour obtained over 1 week. After 2 weeks of T_4 injection the oxygen consumption had reached the level of 101.3 ml., indicated by line (2). Arrow A gives the rise as 18.4 ml. O_2 . Then 3,3',5'-triiodothyropropionic acid (3,3',5'- Pr_3) was superimposed at 1.64 mg./kg./day (a molar ratio 100 times the T_4 dosage).

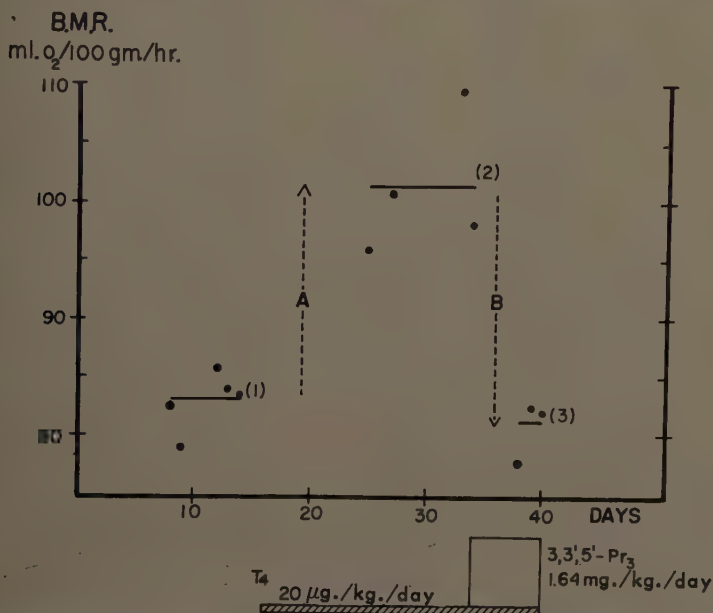


FIGURE 3. Diagram of quantitation of response of thyroidectomized rat B.M.R. to T_4 20 μ g./kg./day (arrow A), and to 3,3',5'- Pr_3 in addition to T_4 (arrow B). The 1.64-mg. 3,3',5'- Pr_3 /kg./day yielded a molar ratio of 100:1 over the 20 μ g. T_4 /kg./day. Adapted by permission of the *American Journal of Physiology*.¹³

B.M.R. the last 3 of 6 days of injection averaged 81.0 ml. O_2 , a drop of 20.3, indicated by arrow B. Inhibition of T_4 effect calculated from arrows B/A $\times 100$ was 105.4 per cent.

In FIGURE 4 are shown the inhibitory effects on T_4 -maintained metabolic rates produced by 4 partially iodinated thyronines injected at inhibitor-to- T_4 molar ratios of 50:1 to 200:1. At 50:1, 3,3',5'- T_3 depressed metabolism only 7 per cent, but completely returned it to the pre- T_4 level at 100:1, with no further decrease at 200:1. The diiodinated compound, 3,3'- T_2 , was approximately as effective as 3,3',5'- T_3 . In contrast, its isomer, 3,5- T_2 , gave 29 per cent inhibition at a molar ratio of 50:1 and only 15 per cent at 100:1. This suggests that 3,5- T_2 is not effective in blocking metabolic actions of T_4 , although a higher dosage might have been effective, as it was the 3-monoiodo-tyronine (3- T_1).

A similar series of trials with 4 analogues with fatty acid side chains was run (FIGURE 5). The results with 3,3',5'-Pr₃ were quite like those with 3,3',5'-T₃, except that an unexpected fall-off was seen with the former at a molar ratio of 200:1. Recovery of complete blockade occurred with a still higher ratio. At the inhibitor-to-T₄ ratio of 100:1, 3,3',5'-Pr₃ and 3,3'-diiodothyropropionic acid (3,3'-Pr₂) were completely effective, 3,3',5'-triiodothyroacetic (3,3',5'-Ac₃) and 3,3'-diiodothyroacetic (3,3'-Ac₂) acids being 62 and 44 per cent inhibitory, respectively. This lower inhibitory activity on the part of the acetic acid analogues may be related to our observation that Ac₄ and 3,5,3'-Ac₃ are somewhat more effective in stimulating metabolism than the cor-

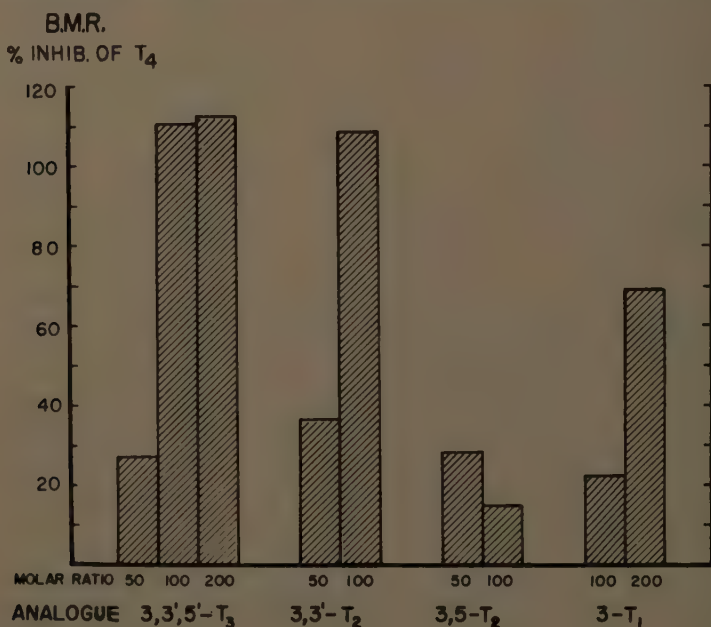


FIGURE 4. Inhibition of thyroxine metabolic effect in thyroidectomized rats. All animals received 20 μ g. T₄/kg./day plus iodothyronine analogue at dosages yielding the indicated molar ratios of analogue:thyroxine. Adapted by permission of the *American Journal of Physiology*.¹³

responding Pr analogues. It should be remarked again that none of the 3,3'-disubstituted or 3,3',5'-trisubstituted analogues has significant metabolism-stimulating properties.

As checks on the specificity of the T₄-reversing properties of these compounds sodium iodide, 3,5-diiodotyrosine, thyronine, and tetranitrothyronine were evaluated at fairly high molar ratios. As TABLE 1 shows, none of these was more than 11 per cent effective against T₄.

To determine the effectiveness of some of the analogues against 3,5,3'-T₃ several groups of animals were injected with this compound at 5 μ g./kg./day. As with T₄, when the B.M.R. had been stabilized at the elevated value, the material to be tested was injected, in addition to the 3,5,3'-T₃. Evaluation of any inhibition of metabolic rate was carried out as before. FIGURE 6 shows

that 3,3',5'-Pr₃ was equally effective against T₄ and 3,5,3'-T₃, while the other 2 substances were appreciably less inhibitory to T₃ than to T₄. At all 3 molar ratios of 50:1, 100:1, and 200:1, 3,3',5'-T₃ was only about one half as active. The 3,3'-T₂ at a ratio of 200:1 was one third less effective. It should be remembered that the metabolism-stimulating 3,5,3'-T₃ dose was only one fourth that of T₄, so that doses of inhibitors were also one fourth, to keep the molar ratio calculations consistent.

B.M.R.

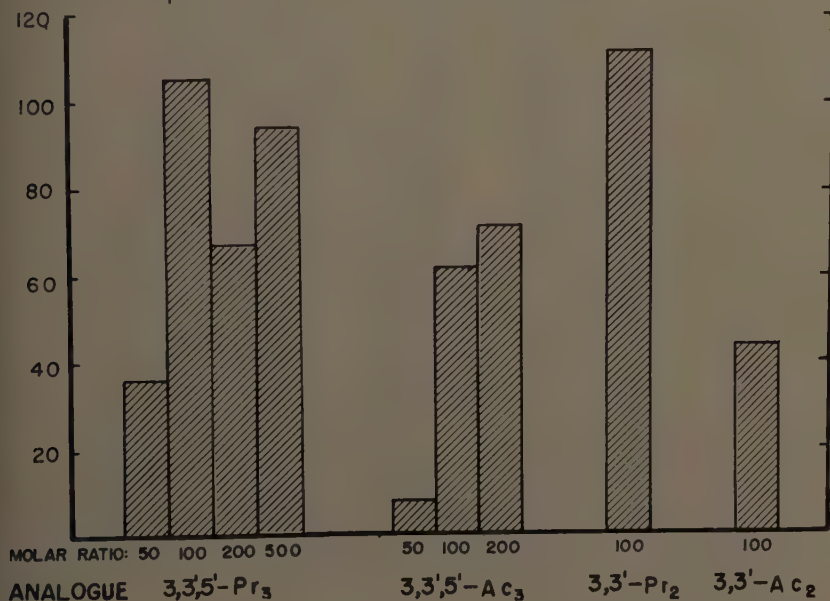
% INHIB. OF T₄

FIGURE 5. Inhibition of thyroxine metabolic effect in thyroidectomized rats. Details are as in FIGURE 4, but with other analogues. Adapted by permission of the *American Journal of Physiology*.¹³

TABLE 1
COMPOUNDS INEFFECTIVE AS THYROXINE INHIBITORS

Compound	Ratio*	B.M.R.† (ml. O ₂ /100 gm./hr.)			Inhibition (%)
		Control	T ₄ ‡	T ₄ + compound	
Sodium iodide	600	84.0	110.8	107.9	10.8
3,5-Diiodo-L-tyrosine	250	77.7	92.1	95.6	0
L-tyrosine	250	84.0	110.3	108.6	6.5
3,5,3',5'-Tetranitro-DL-tyrosine	200	81.5	98.4	101.1	0

* Molar ratio of compound:thyroxine.

† Basal metabolic rate.

‡ Thyroxine.

Effects on Tissue Metabolism

We have made some inquiry into tissue metabolism responses of thyroidectomized rats following injection of T_4 alone and in conjunction with various dosages of 3,3',5'- T_3 . FIGURE 7 shows the oxygen consumption of diaphragm, liver, heart, salivary gland, and kidney from thyroidectomized animals (at least 6 weeks previously) injected with "blank" (alkali plus saline) or with 0.25 mg. T_4 /kg./day for 4 days. Although this dose of hormone was much larger than that used in the whole-animal metabolism experiments described above,

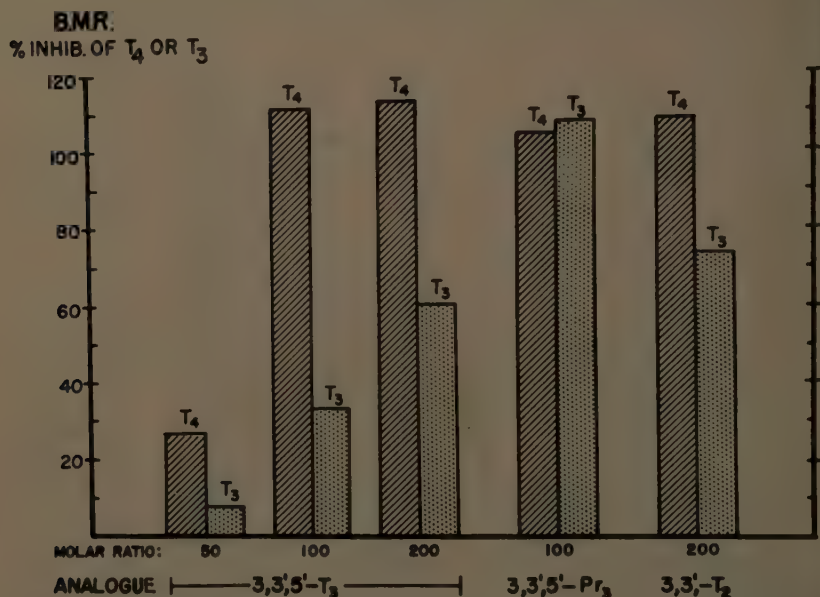


FIGURE 6. Comparison of inhibition of B.M.R. of thyroidectomized rats maintained as indicated on either T_4 or 3,5,3'- T_3 at dosages of 20 or 5 μ g./kg./day, respectively. The figure below each pair of bars represents the molar ratio of analogue to thyroactive compound used for maintenance (either T_4 or T_3). Adapted by permission of the *American Journal of Physiology*.¹³

it was as small as was compatible with a consistent effect, in order to stay within reasonable limits of amount of inhibitor to be used.

The percentage increase over the control for each tissue is shown as the first part of FIGURE 8, including a column (Av.) of 17.5 per cent as an average for the 5 tissues. It is realized that such an unweighted average has certain disadvantages, particularly as a spread develops among some of the responses.

Another group of similar animals was subjected to the same T_4 treatment for 4 days plus an injection of 10 mg. 3,3',5'- T_3 /kg. twice each day. A considerably more erratic response was obtained, especially from diaphragm and salivary gland, but the average was again +18 per cent. When the same total dosage of 3,3',5'- T_3 (20 mg./kg./day) was administered in a single daily injection, the average tissue metabolism increase was 10 per cent, 42 per cent less than with T_4 alone.

In the fourth series shown in FIGURE 8 the animals were given 20 mg. 3,3',5'-T₃/kg./day for 2 days prior to combining it with the 0.25-mg. treatment with T₄. This produced a 62 per cent blockade of the average metabolic increase anticipated from the T₄. The effects were most marked with liver and heart, both of which remained at the untreated hypothyroid level. Pretreatment with 3,3',5'-T₃ might prove even more effective if carried out for more than 2 days.

From these experiments it appears likely that there is a critical concentration of 3,3',5'-T₃ that must be maintained for a certain length of time for effective blocking of T₄ metabolic action. It is known that the rate of disappearance

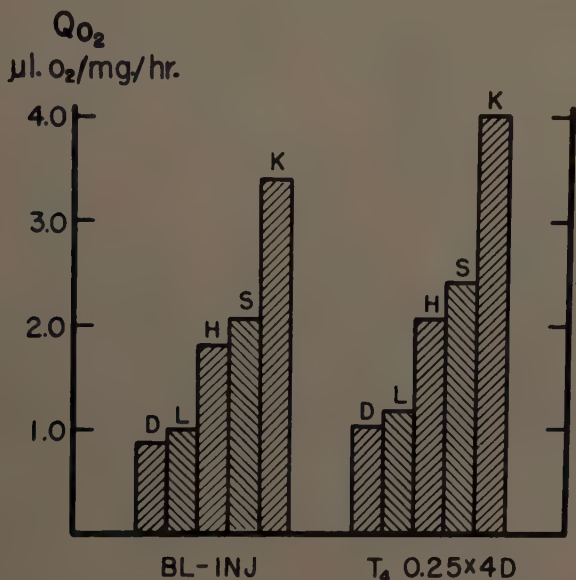


FIGURE 7. Oxygen consumption of tissues from thyroidectomized rats injected with alkaline saline (blank injection, BL-INJ) or with 0.25 mg. T₄/kg./day for 4 days and sacrificed on the fifth day. Tissues are diaphragm (D), liver (L), heart (H), salivary gland (S), and kidney (K).

of 3,3',5'-T₃ from plasma after injection is very much more rapid than that of T₄ or 3,5,3'-T₃. For this reason we tried the divided dose technique, which did not prove effective at the dose-time combinations employed here.

Human Metabolism Studies

Two series of patients were studied, one with 3,3',5'-T₃ and the other with 3,3',5'-Pr₃. There has been a clear indication of reversal of T₄ and 3,5,3'-T₃, and of desiccated thyroid metabolic effects with 3,3',5'-T₃ but, in contrast to the animal work, 3,3',5'-Pr₃ at comparable dose levels has been without effect on either B.M.R. or serum cholesterol levels.

Of 4 patients with myxedema being treated with various types of replacement therapy, 3 exhibited 3,3',5'-T₃-antagonistic effects on the B.M.R. FIGURE 9 plots the treatment of patient W.M. with 100 μg. 3,5,3'-T₃/day, which brought

his B.M.R. from -31.8 to -12.0 per cent. When 110 mg. $3,3',5'$ - T_3 (molar ratio about $1200:1$) were added for 8 days, the B.M.R. was depressed to -24.3 per cent. A few days after withdrawal of the $3,3',5'$ - T_3 the B.M.R. had returned to -13.3 per cent, close to the previous $3,5,3'$ - T_3 -maintained level. Using the average of the 2 series of $3,5,3'$ - T_3 B.M.R. periods, a 61 per cent reversal was achieved by the $1200:1$ molar ratio of inhibitor: $3,5,3'$ - T_3 .

In the case of patient R.C., maintained at a B.M.R. of -8.4 per cent, up from -24.4 per cent, on 300 μ g. T_4 /day, addition of 80 mg. $3,3',5'$ - T_3 /day low-

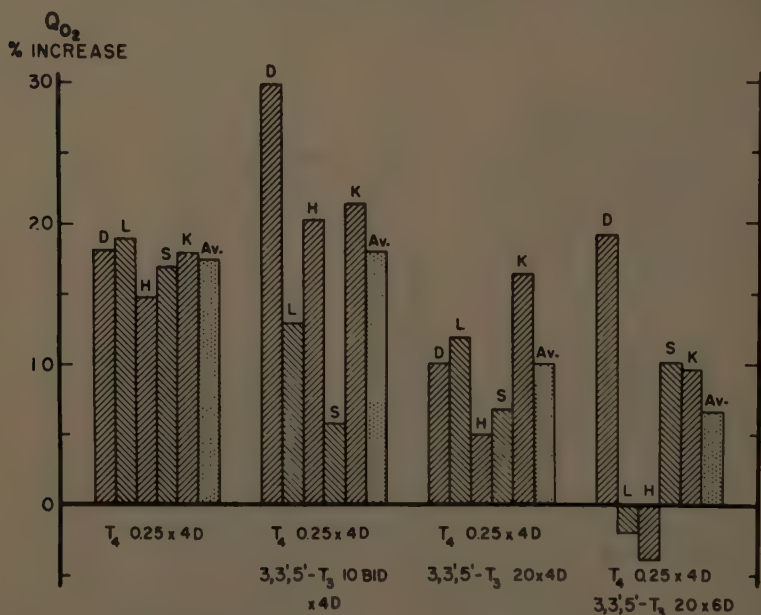


FIGURE 8. Oxygen consumption of tissues from thyroidectomized rats injected with T_4 alone or in various combinations with $3,3',5'$ - T_3 . Values are plotted as percentage of increase above control injected tissue metabolism (FIGURE 7). Vertical bars represent the same tissues plus an unweighted average (stippled and labeled *Av.*). All 4 groups contained 3 animals each, all injected with 0.25 mg. T_4 /kg./day for 4 days and sacrificed on the fifth day. The group represented at the left received only T_4 and the others, in order, 10 mg. $3,3',5'$ - T_3 /kg. twice a day for the same 4 days, 20 mg. $3,3',5'$ - T_3 /kg. once a day, and 20 mg. $3,3',5'$ - T_3 /kg. once a day, starting 2 days prior to the administration of T_4 and continuing throughout.

ered the B.M.R. to -12.5 per cent. Further increase of $3,3',5'$ - T_3 dosage to 160 mg./day yielded the still lower B.M.R. of -17.0 per cent. As shown in FIGURE 10, these 2 molar ratios were calculated out to $320:1$ and $640:1$, producing T_4 reversals of 26 per cent and 54 per cent, respectively. It is interesting that the $640:1$ molar ratio effected about the same degree of metabolic interference with T_4 as did the $1200:1$ ratio in the patient with $3,5,3'$ - T_3 . This is similar to what we observed with thyroidectomized rats on T_4 and $3,5,3'$ - T_3 , as shown in FIGURE 6, although the amounts of inhibitor in proportion to thyroactive substance required by the humans were much higher than with rats.

Patient S.M., with myxedema, maintained on 120 mg. desiccated thyroid,

displayed (FIGURE 11) an increasing return of hypometabolism at 80 and 160 mg. $3,3',5'-T_3$ /day. A pretreatment B.M.R. estimate of -35 per cent allows the calculation of a 22 per cent reversal of T_4 effect at the 80-mg./day dosage of $3,3',5'-T_3$, and an 89 per cent reversal at 160 mg., as shown in FIG-

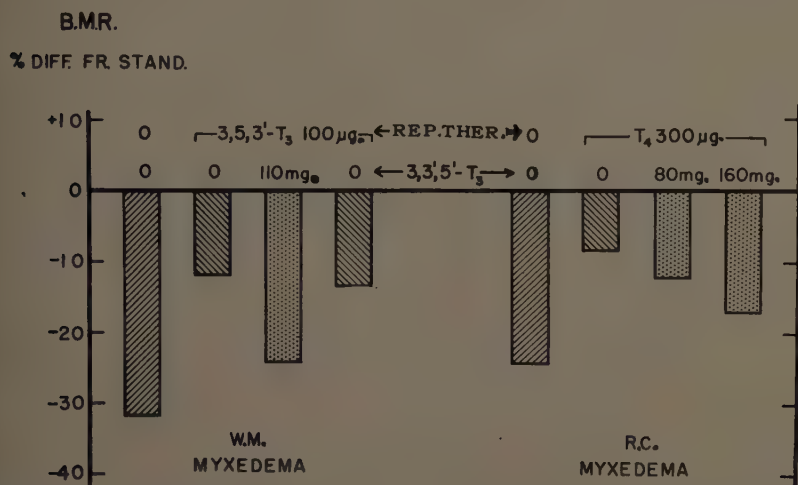


FIGURE 9. Effect of $3,3',5'-T_3$ on B.M.R. of myxedematous human patients maintained on $3,5,3'-T_3$ or on T_4 . Ordinate shows B.M.R. in the usual clinical terms of percentage of difference from standard values. At the left, vertical bars for W.M. indicate, in order, B.M.R. with no therapy, $3,5,3'-T_3$ 100 µg./day, $3,5,3'-T_3$ plus 110 mg. $3,3',5'-T_3$ per day, and return to only $3,5,3'-T_3$ 100 µg./day. The bars for R.C. show off-treatment B.M.R., replacement with 300 µg. T_4 /day, then T_4 plus 80 mg. $3,3',5'-T_3$ per day and, last, T_4 plus 160 mg. $3,3',5'-T_3$.

B.M.R.

% inhib. of rep. ther.

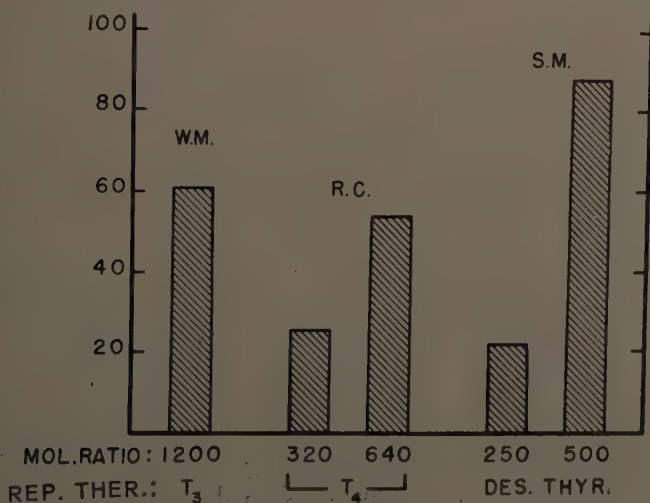


FIGURE 10. Effect of $3,3',5'-T_3$ on B.M.R. of 3 myxedematous human patients (see FIGURES 9 and 11), recalculated as inhibition of replacement therapy. See text for details.

FIGURE 10. The molar ratios shown for patient S.M. in FIGURE 10 were obtained by equating the physiological responses to 120 mg. of desiccated thyroid and 300 μ g. of T_4 .

The other myxedematous patient, F.S., was only partially controlled (FIGURE

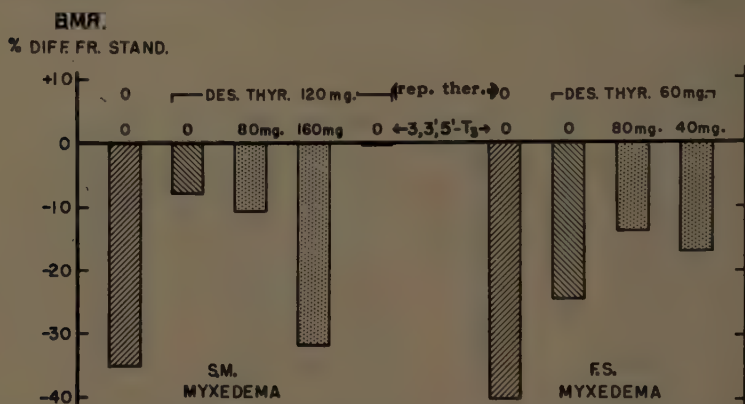


FIGURE 11. Effect of 3,3',5'-T₃ on B.M.R. of myxedematous human patients treated with desiccated thyroid. Ordinate as in FIGURE 9. Dosages of both desiccated thyroid and 3,3',5'-T₃ given in mg./day.

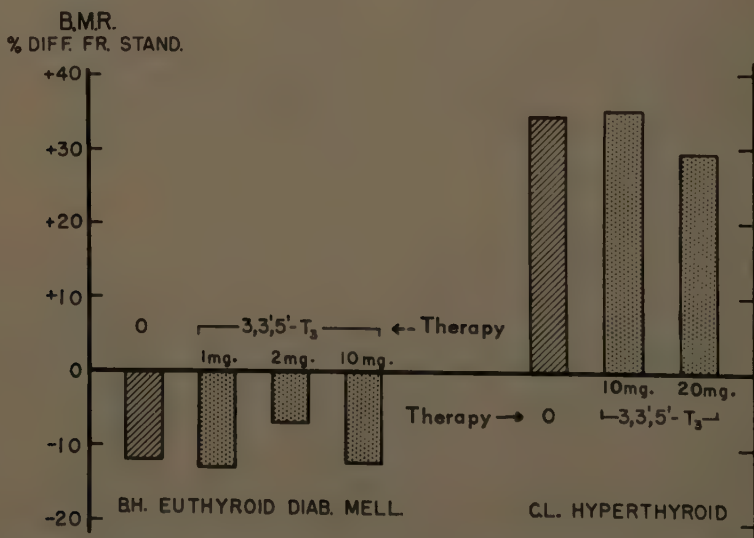


FIGURE 12. Effect of 3,3',5'-T₃ on B.M.R. of 1 euthyroid and 1 thyrotoxic patient. Ordinate as in FIGURE 9. Dose levels of 3,3',5'-T₃ shown are in mg./day.

11) on 60 mg./day of desiccated thyroid and, during added treatment with both 40 and 80 mg. of 3,3',5'-T₃ the B.M.R. rose slightly. In the absence of a final period of being on desiccated thyroid only, it is possible to explain the findings on a basis of inadequate stabilization with the 60-mg. dose.

One euthyroid patient, B.H., received up to 10 mg. of 3,3',5'-T₃ for 13 days (FIGURE 12) with no change in his B.M.R. The absence of a rise is in line with

the lack of thyroidlike effect of 3,3',5'-triiodo compounds on the metabolism of thyroidectomized rats. The lack of fall in B.M.R. is not surprising in view of the dose levels of inhibitor required by the previously discussed myxedematous patients maintained on exogenous hormone.

FIGURE 12 also shows that C.L., a hyperthyroid patient with a B.M.R. of +34 per cent, was not relieved by 10 or 20 mg. 3,3',5'-T₃/day. If it is estimated that the hyperactive gland was secreting at least twice as much hormone as is needed by a myxedematous patient, or $2 \times 300 \mu\text{g. T}_4/\text{day}$, the larger dose of 20 mg. 3,3',5'-T₃ would yield a molar ratio of only 30:1. This is somewhat less than the effective ratio of 100:1 obtained in rats (FIGURE 4) and is far below the range of 250:1 to 1200:1 ratios already found necessary in the human

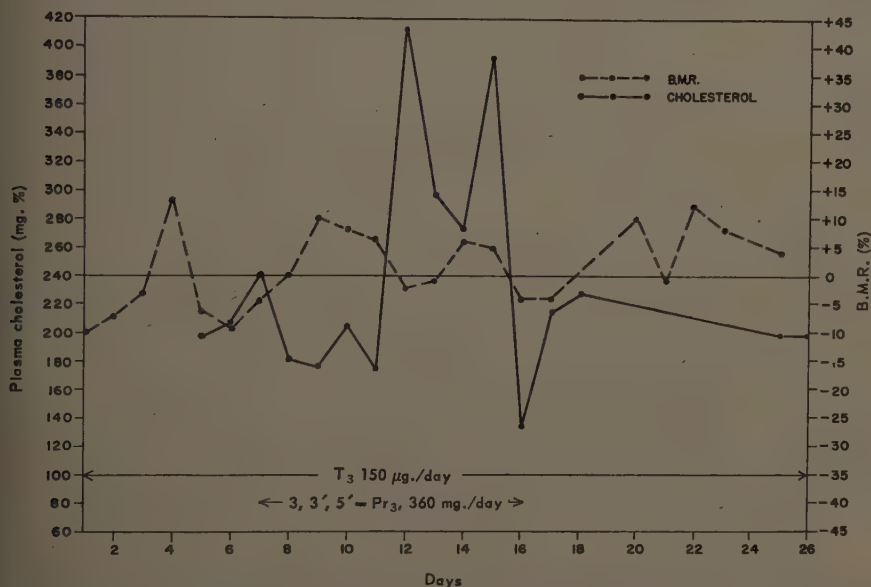


FIGURE 13. Plot of B.M.R. and serum cholesterol values for R.W.C., a myxedematous patient, on 3,5,3'-T₃ throughout, plus 3,3',5'-Pr₃ from days 7 through 16. Dosages were administered orally, once a day.

(FIGURE 10). Benua *et al.*,⁴ in fact, have been able to lower the B.M.R. of a hyperthyroid patient from +48 to +13 per cent during 12 days' administration of 120 mg. 3,3',5'-T₃/day.

A smaller series of studies has been carried out using 3,3',5'-triiodothyropropionic acid in man. In contrast to both animal studies with 3,3',5'-Pr₃ and the human studies with 3,3',5'-T₃, the propionic analogue had no apparent effect on either B.M.R. or blood cholesterol levels. FIGURE 13 shows the day-to-day fluctuations of these indexes in R.W.C., a myxedematous patient receiving 150 $\mu\text{g. T}_3/\text{day}$ before, during, and after 360 $\mu\text{g. 3,3',5'-Pr}_3/\text{day}$. Even with the 2 noticeably high serum cholesterol values during combined therapy, no consistent trend is present. Patient S.M., shown in FIGURE 11 to respond to 3,3',5'-T₃ while on desiccated thyroid, is seen in FIGURE 14 to be resistant to 3,3',5'-Pr₃, although it was administered at a considerably higher dose in relation to thyroid therapy than was the 3,3',5'-T₃. Similarly, no 3,3',5'-Pr₃ change

was seen in J.C., a euthyroid orthopedic patient, placed at the outset of the study on methimazole, to prevent any rise in endogenous thyroid hormone production (FIGURE 15).

In the same patients, R.W.C., S.M., and J.C., the rates of disappearance of

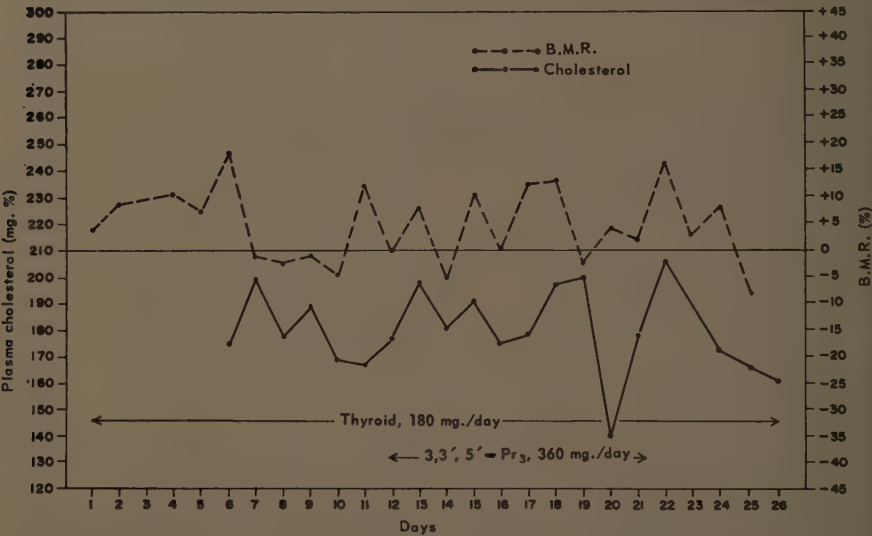


FIGURE 14. B.M.R. and serum cholesterol values for S.M., patient with myxedema treated with desiccated thyroid throughout, given 3,3',5'-Pr₃ from days 12 to 21. Dosages shown were administered orally, once a day.

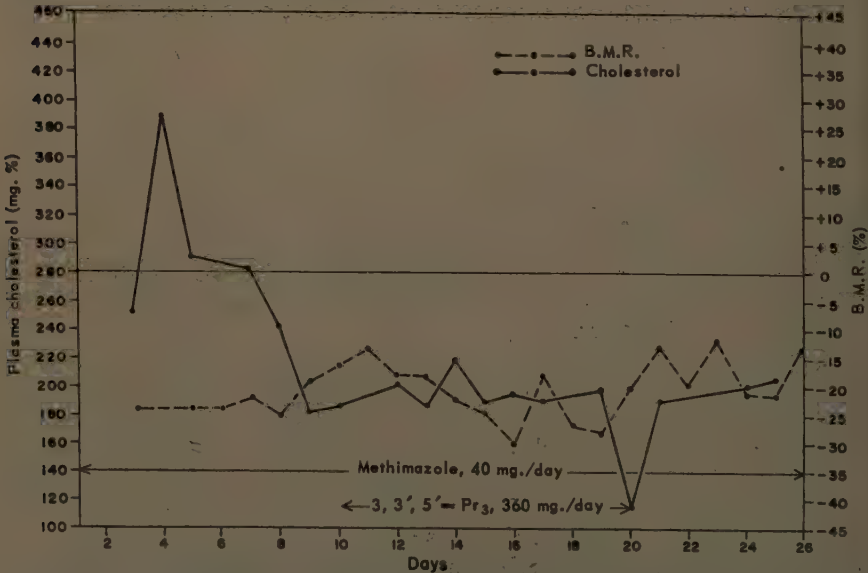


FIGURE 15. B.M.R. and serum cholesterol values for J.C., euthyroid patient receiving 40 mg. methimazole per day. From days 10 through 20 treated with 360 mg. 3,3',5'-Pr₃, as shown.

intravenously injected tracer doses of radioactive T_4 remained unaltered during the administration of 3,3',5'- Pr_3 .

Antigoitrogenicity Studies

One of the widely employed tests of thyromimetic activity is the prevention of thiouracil-induced thyroid gland enlargement. Since these goiters do not appear in hypophysectomized animals placed on thiouracil, it is implied that the principal antigoitrogenic action is exerted on the pituitary, blocking release of thyrotropin (TSH). The idea has occurred to others that it should be possible to evaluate interference with T_4 action by measuring interference with its antigoitrogenic effect. Cortell⁵ showed in 1949 that 2',6'-diiodothyronine reversed T_4 antigoitrogenesis, but Plamondon *et al.*⁶ later found it to have no effect on the metabolism-supporting function of T_4 .

TABLE 2
ANTIGOITROGENIC EFFECT OF THYROXINE AND ITS REVERSAL
BY 3,3',5'-TRIIODOTHYROPIONIC ACID*

Treatment	Thyroid weight (mg./kg.)	Treatment	Molar ratio†	Thyroid weight (mg./kg.)	T_4 effect (%)
MIZ 0.1% in water 12 days	157	PTU 0.1% in food 11 days	—	200	100
+ 3,3',5'- Pr_3 819†	156	+ 3,3',5'- Pr_3 1638	—	203	
+ 3,3',5'- Ac_3 801	159	+ T_4 5	—	153	
+ 3,3',5'- T_3 838	157	+ T_4 10	—	120	
Controls	54	+ T_4 15	—	108	
		Diet without PTU	—	61	
PTU 0.1% in water 13 days	181	PTU + T_4 10			101
+ 3,3',5'- Pr_3	177	+ 3,3',5'- Pr_3 410	50	119	
Controls	64	+ 3,3',5'- Pr_3 819	100	146	
		+ 3,3',5'- Pr_3 1229	150	158	

* Abbreviations: T_4 = thyroxine; Pr_3 = triiodothyropropionic acid; Ac_3 = triiodothyroacetic acid; T_3 = triiodothyronine; MIZ = methimazole; PTU = propylthiouracil.

† Molar ratio of 3,3',5'- Pr_3 : T_4 .

‡ All doses are given as $\mu\text{g.}/\text{kg.}/\text{day}$ for the number of days shown.

Obviously, in order to possess measurable T_4 -blocking action, any compound evaluated by this test should itself have no detectable pituitary-depressant effect. There is contradiction in the recent literature. Roche *et al.*⁷ report temporarily complete blocking of thyroidal organic iodine release by 3,3',5'- T_3 and Money *et al.*⁸ concur, with a somewhat more complex test of TSH activity, while Stasilli *et al.*² report a complete lack of antigoitrogenic action of 3,3',5'- T_3 . Our experience has been that no decrease in thyroidal gland size of rats on methimazole or propylthiouracil has resulted from injection of 3,3',5'-triiodinated compounds up to the equivalent of 1000 or 2000 $\mu\text{g. } T_4/\text{kg.}/\text{day}$ for 11 to 13 days, as shown in TABLE 2. In addition, it can be seen from FIGURES 16, 17, and 18 that no change has been seen in discharge rate of radioiodine from the methimazole-blocked thyroid glands of rats injected with single doses of up to 200 $\mu\text{g. } 3,3',5'-T_3$ or 500 $\mu\text{g. } 3,3',5'-Pr_3$, in contrast to prompt arrest with T_4 or 3,5,3'- T_3 . (Michel has stated that he had obtained similar results with propylthiouracil-thyroids, in contrast to those with animals not treated

with goitrogen.⁷ One facile but somewhat implausible explanation is that $3,3',5'$ - T_3 enhances reincorporation of iodide by the thyroid.)

With these assurances of no $3,3',5'$ -triiodo interference via the pituitary, we can examine the antigoitrogenic data obtained with T_4 alone and in conjunction with $3,3',5'$ - Pr_3 (TABLE 2). At a molar ratio of $3,3',5'$ - Pr_3 to T_4 of 50:1, there was no interference with antigoitrogenesis; at 100:1 and 150:1 the 10- μ g. dose of T_4 was only 68 and 53 per cent as effective. Thus, there was a

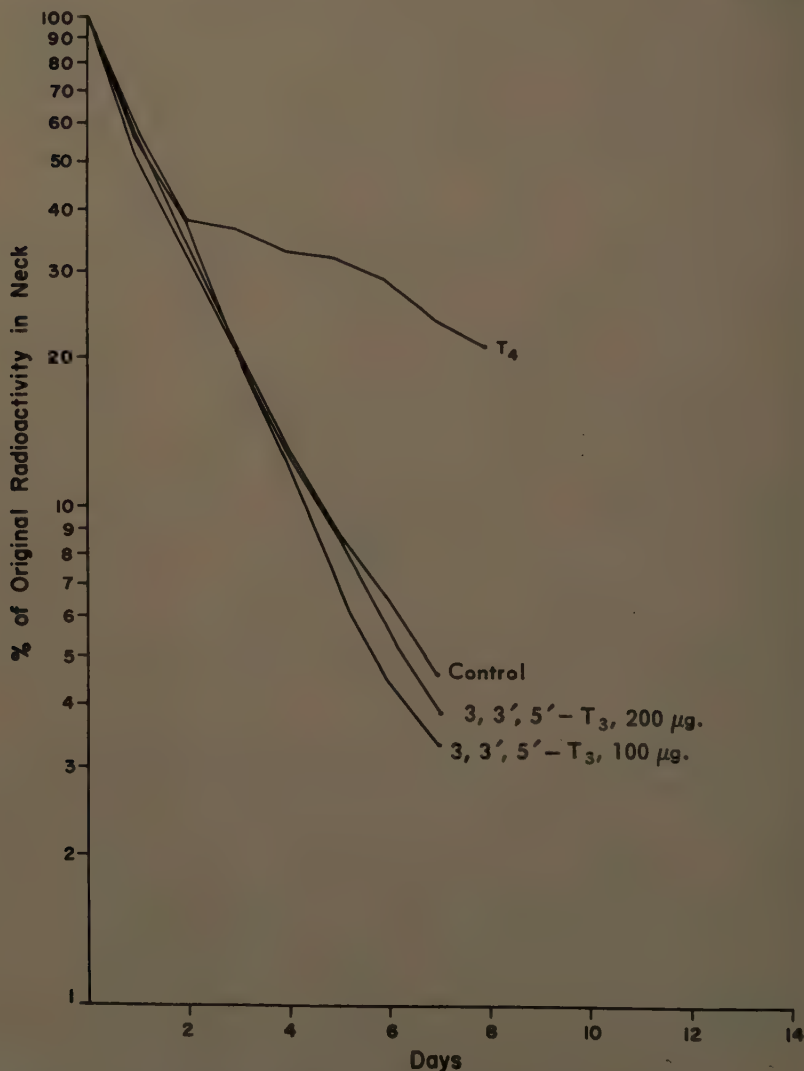


FIGURE 16. Comparison of effects of T_4 and $3,3',5'$ - T_3 on I^{131} discharge rate from thyroid region of rats. Animals were given radioiodide one day before the "zero" day, when methimazole (0.1 per cent in the food) was started, to block reincorporation of inorganic iodide. A single dose of 100 μ g. T_4 , 100 μ g. $3,3',5'$ - T_3 or 200 μ g. $3,3',5'$ - T_3 was injected on day 2. Each group was made up of 8 animals.

32 to 47 per cent depression of T_4 action on the pituitary, although less than would be expected on the basis of the previous oxygen consumption results.

Attempts at in Vitro Inhibition

In order to determine whether the T_4 -blocking actions are explainable on a purely peripheral basis, we have explored 2 *in vitro* systems capable of responding to T_4 . These are long-term maintenance of kidney cortex oxygen consumption and enhanced response of aorta smooth muscle to applied epinephrine.

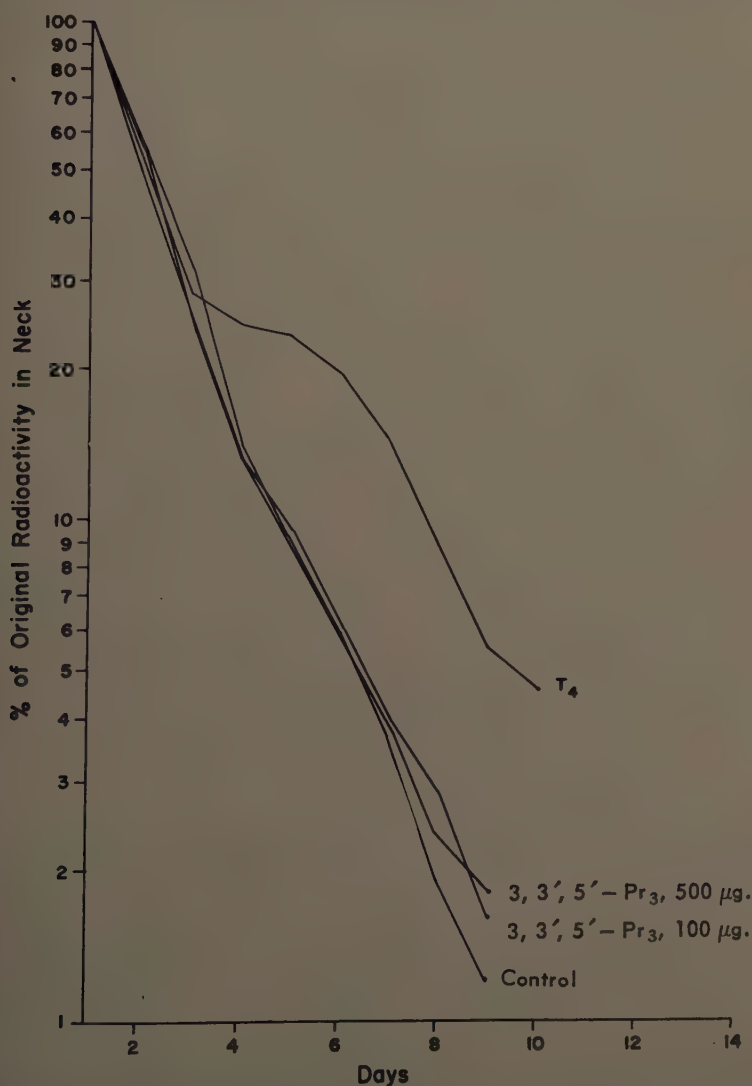


FIGURE 17. Comparison of effects of T_4 and $3,3',5'-Pr_3$ on thyroïdal I^{131} discharge rate. Radioiodide and methimazole as in FIGURE 16. A single injection of 40 $\mu g.$ T_4 , 100 $\mu g.$ $3,3',5'-Pr_3$, 500 $\mu g.$ $3,3',5'-Pr_3$, or saline was given on day 3.

Although both are less sensitive to the 3,3',5'-triiodo analogues, there is sufficient residual responsiveness to interfere with any clear-cut inhibitory action.

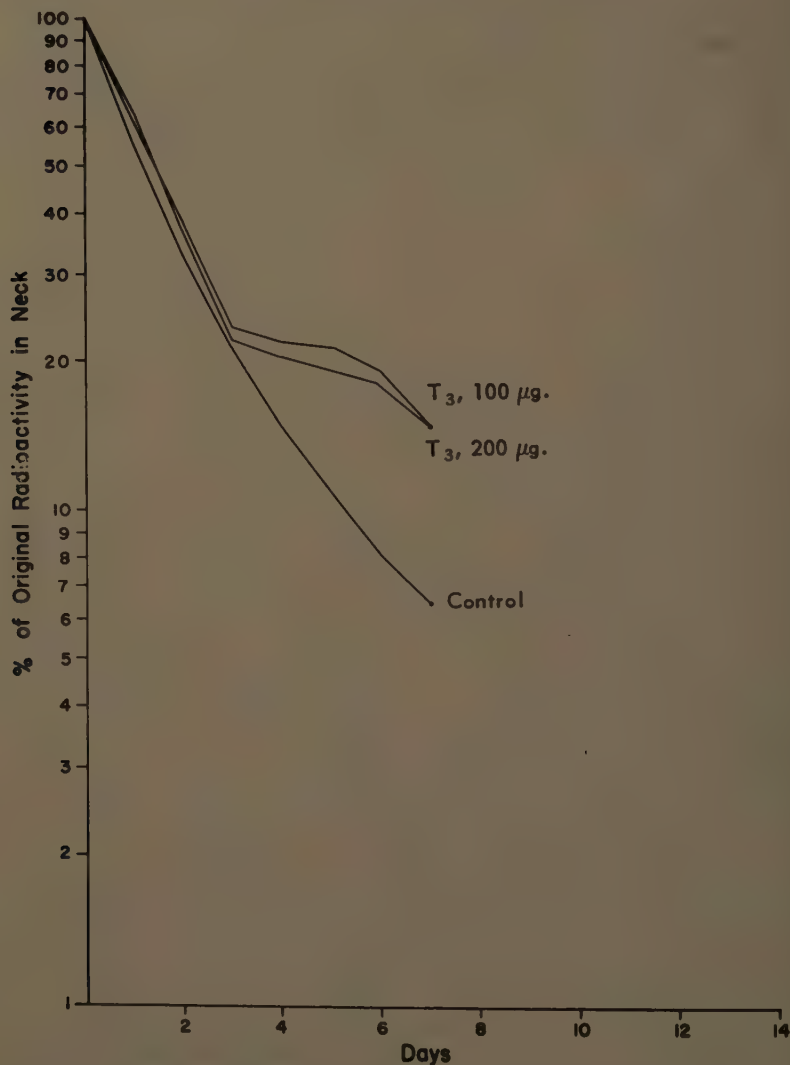


FIGURE 18. Effects of 3,5,3'-T₃ on thyroïdal I¹³¹ discharge rate. Radioiodide and methimazole as in FIGURE 16. A single injection of 100 µg. T₃ or saline was made on day 3.

General Discussion

A marked difference between responsiveness of the rat and the human to the propionic acid analogues has been shown in the present study. This strongly suggests a considerably greater side-chain specificity in the response of man to T₄ and T₃ blocking agents than in that of the rat. Such a difference has been evident for some time in terms of the metabolism-stimulating action of

the acetic and propionic acid analogues⁹ of T_4 and T_3 . In all instances the human is far less responsive than the rat. It may be noted parenthetically that these inhibiting 3,3'-diiodo- and 3,3',5'-triiodo-substituted compounds should be evaluated in the metamorphosing amphibian, where a superiority of propionic and acetic acid analogues over T_4 and T_3 has already been encountered.^{10,11}

When the experimental animals were returned to hypometabolism by treatment with one of the analogues studied, other signs of hypothyroidism, such as sluggishness in response, weight gain, coarsening of skin and hair, and accumulation of epicanthal exudate, were evident. Similar signs of general blockade of peripheral actions of T_4 were not seen during the short-term experiments with the human patients. Comparable degrees of hypothyroidism apparently were not maintained for sufficient time.

It is intriguing to interpret the well-known latency of response to T_4 as attributable to the time required for some intracellular reorientation to take place, perhaps resulting in a greater amount of enzymically active protein. On this basis, the presence of even such rapidly excreted analogues as 3,3',5'- T_3 or 3,3'- T_2 at the critical time could prevent the T_4 triggering.

It is highly questionable, and not yet settled, that such T_4 -inhibiting agents will prove to have any genuine therapeutic usefulness in man. We do not wish to imply any such possibility at the present time. From the physiological point of view it is more interesting to pursue the possibility that 3,3',5'- T_3 and 3,3'- T_2 might serve as endogenously produced thyroid hormone regulators. Both of these substances have been identified in the plasma of rats,¹² although in very small amounts. No quantitative estimate is available, but it seems doubtful that any such ratio of these substances as 50:1 or 100:1 to T_4 or even to T_3 would be reached under ordinary conditions. Such a situation may be encountered as a metabolic aberration.

Experience in this laboratory leads us to believe that partial deiodination of T_4 or T_3 takes place much less extensively than complete deiodination or deamination plus decarboxylation of the alanine side chain. Unless production of 3,3',5'- T_3 or 3,3'- T_2 takes place intimately related to any site of action of T_4 or 3,5,3'- T_3 , regulatory action probably would be difficult.

Summary

3,3'-Diiodothyronine, 3,3',5'-triiodothyronine, and their propionic acid analogues have been found to return thyroxine-maintained metabolic rates of thyroidectomized rats to the hypothyroid level. Molar ratios of inhibitor to thyroxine of 50:1 to 200:1 were required. At these dosages, 3,3'-diiodo- and 3,3',5'-triiodothyroacetic acids were less effective.

Tissue metabolism responses following fewer injections of thyroxine at a higher dose level were also depressed when the thyroidectomized animals were treated simultaneously with 3,3',5'-triiodothyronine.

Three myxedematous human patients with basal metabolic rates maintained on thyroxine, triiodothyronine, and desiccated thyroid, respectively, exhibited lowered metabolism when treated with 3,3',5'-triiodothyronine at molar ratios of 500:1 to 1000:1. A fourth patient failed to respond. One euthyroid and one thyrotoxic patient showed no decrease in basal metabolic rate, probably

because of inadequate doses. When 3,3',5'-triiodothyropropionic acid was used, no changes were observed in basal metabolic rates or serum cholesterol levels, even though higher molar ratios of analogue to thyroactive substance were employed.

No antigoitrogenic action of 3,3',5'-triiodothyronine or of its propionic and acetic acid analogues has been seen in propylthiouracil- or methimazole-treated rats. The 3,3',5'-triiodothyronine blocked up to 47 per cent of thyroxine antigoitrogenesis.

These results are discussed in terms of possible endogenous and exogenous control of thyroid hormone action.

Acknowledgments

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METABOLIC EFFECTS OF THYROXINE ANALOGUES IN HUMAN MYXEDEMA*

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The increased availability of thyroxine analogues in the past 10 years has stimulated investigation of their biological effects. Some recent observations made with analogues in humans are presented in this paper and discussed, together with a review of present knowledge of their metabolic effects. The term thyroxine analogues, as used here, includes any compound containing the diphenoxy- (or diphenothio-) nucleus (FIGURE 1) with substitutions in the R, R', X and X' positions. Thyroid hormones presently established for man, that is, L-thyroxine¹⁻³ and 3:5:3'-L-triiodothyronine,^{4,5} are not considered analogues. The hormones will be considered in the review only in so far as they are necessary standards of reference. The effects of some analogues in human myxedema have been reviewed elsewhere^{6,7} most recently by Selenkow and Asper in 1954.⁸

Methods

The analogues used in the studies reported in this paper were furnished by the Warner-Lambert Research Institute, Morris Plains, N. J. Analogues given by the oral route were in tablet form. Patients were studied in the outpatient clinics of Memorial Center or on the metabolic ward of the James Ewing Hospital, New York, N. Y. The B.M.R. method was that of Roth⁹ in the routine laboratory, or at the bedside in the case of patient L.N. Labeled 3:5:3'-triiodothyropropionic acid (T₃ Prop) with I¹³¹ in the 3' position was obtained from Abbott Laboratories, North Chicago, Ill. It was dissolved in 50 per cent propylene glycol and was contaminated by about 5 per cent radioiodide, according to chromatographic analysis by the manufacturer.

Serum cholesterol was measured by a modification of the Schoenheimer-Sperry technique,¹⁰ and the serum protein-bound iodine and total iodine by a modified chloric acid method.¹¹ Balance studies were performed in collaboration with Olaf Pearson, using methods previously described.¹²

Results

Distribution studies. Little is known concerning the distribution and fate of thyroxine analogues. The purity, stability, solubility, and route of administration, as well as the speed and completeness of absorption, may have strong influences on the results of studies. Although radioiodine labeling

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makes possible the tracing of compounds without interference from pharmacological effects, few labeled analogues have been tested in humans.^{13,14}

Studies of the deiodination of I^{131} -labeled T_3 Prop were carried out in 2 euthyroid patients. Such subjects were selected so that the partition of the radio-

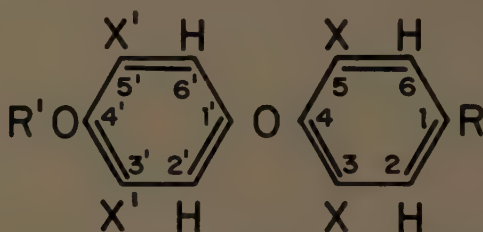


FIGURE 1. The diphenoxy- nucleus of the thyroxine analogues.

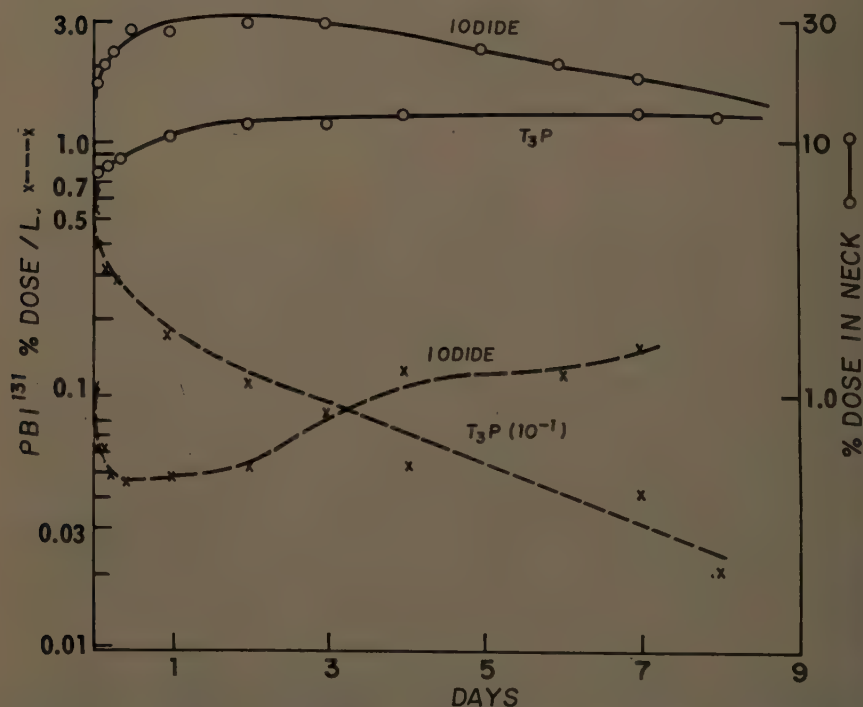


FIGURE 2. A comparison of the uptake of protein-bound and thyroidal radioiodine after the intravenous administration of labeled T_3 Prop and iodide in 2 studies on the same patient. The values for I^{131} iodide are corrected for residual radioactivity from the preceding dose of labeled T_3 Prop. Note that PBI^{131} after T_3 Prop has been divided by 10 before plotting.

iodine between the thyroid and the urine could be compared with the partition after a subsequent radioiodide tracer. Each patient received a dose of 300 μ c. containing about 3 mg. of T_3 Prop.

A comparison of observations following labeled T_3 Prop and a subsequent intravenous sodium radioiodide tracer in 1 patient is made in FIGURE 2. This patient was euthyroid and convalescent from abdominal surgery performed 4

weeks previously. The urine contained 12.1 per cent of the dose at the end of 24 hours and another 5 per cent 48 hours after the administration of the labeled T_3 Prop. At similar intervals following the administration of sodium radioiodide, 33 per cent and 7.5 per cent were excreted in the urine. Similar blood and thyroid curves were obtained in a second euthyroid patient.

In athyreotic patients serial observations of the serum protein-bound iodine (PBI) after a single intravenous injection or after stopping daily administration of the analogues have been used to measure the rates of loss of the compounds from the blood. FIGURE 3 shows such data obtained following the administration of some compounds containing 3 atoms of iodine. After an initial rapid loss of PBI from the blood, occurring only in the data for the intravenous route, a final less rapid rate of loss was recorded. This final phase, measured between 2 and 7 days, decreases at a rate equivalent to a half time

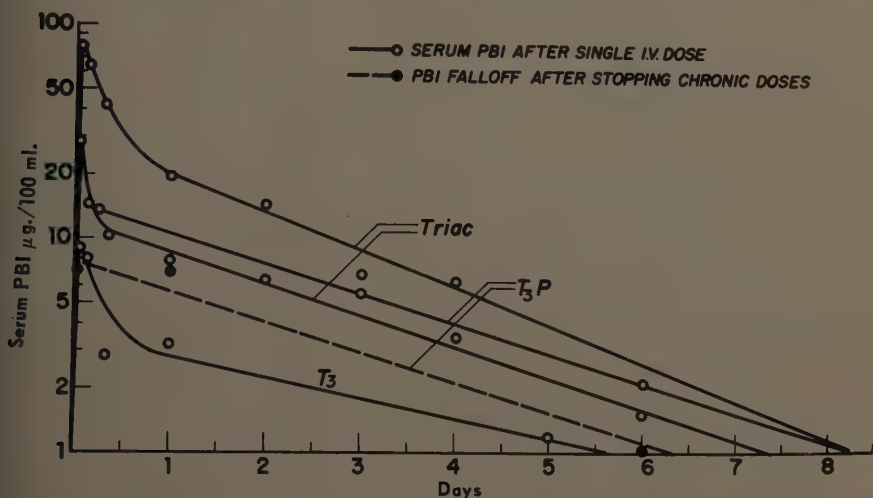


FIGURE 3. Serial PBI data following the administration of certain triiodinated analogues: serum PBI after a single intravenous dose or after termination of chronic oral doses.

of 2 to 3 days for 3:5:3'-L-triiodothyronine (T_3), 3:5:3'-triiodothyroacetic acid (TRIAC), and T_3 Prop. This rate agrees closely with the rate reported for I^{131} -labeled T_3 by Sterling and his co-workers.¹⁵

The PBI decreased at a slower rate, however, after the injection of 3 mg. of L-thyroxine intravenously in G.B., a myxedematous subject,¹⁶ with a rate corresponding to a half time of 6.5 days. This value agrees well with that observed after I^{131} -labeled thyroxine in myxedematous subjects.^{17,18}

The rate of disappearance of 3:3':5'-triiodothyropropionic acid (reverse T_3 Prop) from the serum after chronic administration was different in 2 studies. Patient J.E. had been receiving 400 mg. of reverse T_3 Prop daily for 14 days and 37 µg. of L- T_3 daily for 7 days prior to the time reverse T_3 Prop was discontinued. The PBI decreased slowly, the rate being equivalent to a half time of 14 days, while T_3 was continued. The other patient, B.H., received a single oral dose of 100 mg. of reverse T_3 Prop. A plot of the log of the serum PBI during this study is shown by the first half of the curve in FIGURE 4. The

PBI decreased as rapidly as it had following the other triiodinated compounds shown in FIGURE 3, with a rate corresponding to a half time of 2.5 days.

The percentage of the total serum I^{127} precipitable by trichloroacetic acid was determined in athyreotic patients receiving thyroid hormone or analogues. In 20 athyreotic patients maintained on T_3 Prop, serum was collected 8 to 14

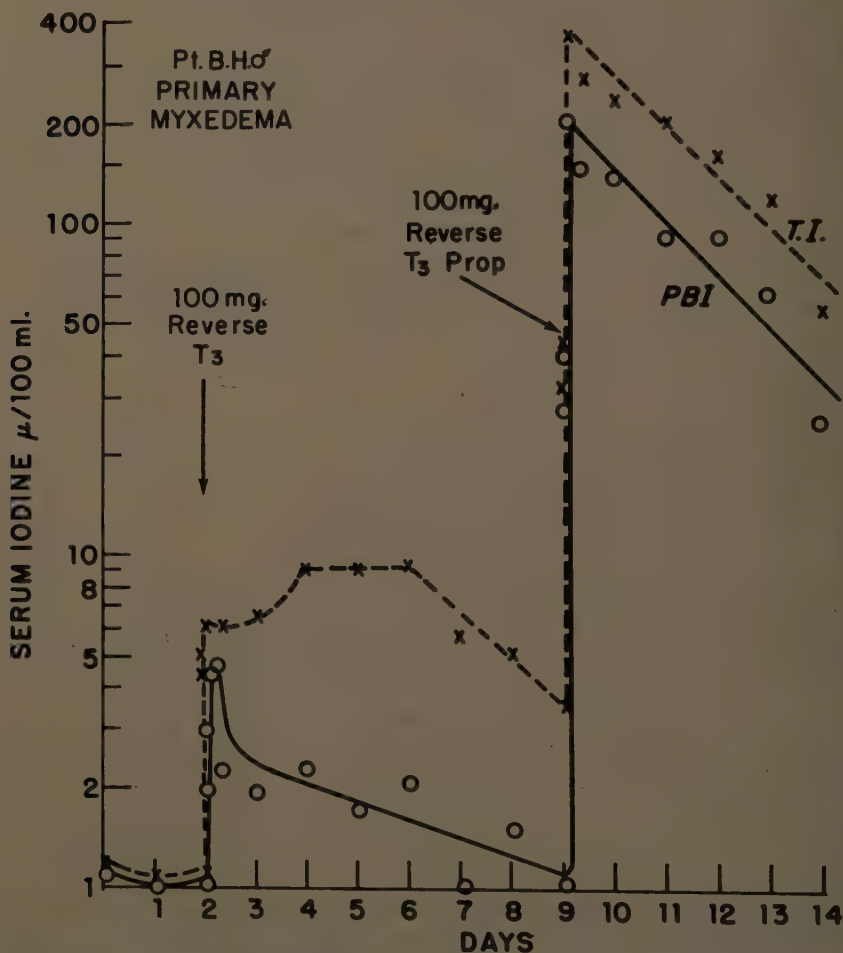


FIGURE 4. Logarithmic plot of PBI and total iodine in serum after single oral doses of $3,3',5'-T_3$ and $3,3',5'-T_3$ Prop (reverse triiodinated analogues).

hours after the last dose. With T_3 Prop the PBI was 60 per cent of the total iodine and was independent of the dose of the analogue, which ranged from 0.2 to 4 mg./day. By comparison, about 90 per cent of the serum iodine was precipitable after the oral ingestion of maintenance doses of sodium L-thyroxine. A similar low percentage of PBI was observed after the intravenous injection of T_3 Prop. In patient L.N., 3 days after the administration of 10 mg. of T_3 Prop, the PBI was 67 per cent of the total iodine. By comparison,

the PBI was 89 per cent of the total iodine 3 days after 15 mg. of TRIAC in patient A.S. and 92 per cent after 20 mg. of tetraiodothyroacetic acid (TETRAC) in patient D.Y.¹⁹ Elevations of the serum total iodine which were out of proportion to the elevations of the PBI followed the administration of 3:3':5'-DL-triiodothyronine (reverse T_3) and reverse T_3 Prop. The oral administration of 100 mg. of each analogue to a patient with myxedema produced the curves for total iodine and PBI shown in FIGURE 4. Three days after the oral administration of reverse T_3 , the PBI was 17 per cent of the total iodine, and 3 days after reverse T_3 Prop it was 58 per cent of the total iodine. The level of total iodine was markedly different, however.

Metabolic effects. T_3 Prop and reverse T_3 Prop were tested for effect on the B.M.R. in human myxedema. In 10 athyreotic patients, the daily oral dose of T_3 Prop necessary to maintain a normal B.M.R. ranged between 1.25 and 5 mg., and averaged 2.5 mg. The intravenous injection of 10 mg. of T_3 Prop in patient L.N. produced an increase in the B.M.R. from -29 to -17 per cent, compared with from -28 to +1 per cent after 0.5 mg. of T_3 . A comparison of areas under the B.M.R. curves after the 2 injections showed 7 times more area above the projected base line following 0.5 mg. of T_3 than after 10 mg. of T_3 Prop. There was no change in B.M.R. during the first 24 hours following the intravenous injection of T_3 Prop, but it had increased significantly 6 hours following the injection of T_3 . Oral reverse T_3 Prop, in daily doses as large as 400 mg., did not increase the B.M.R. of patient J.E. during a 14-day study.

The response of the B.M.R. and the serum cholesterol level were compared in one patient, J.E., during the oral administration of T_3 , T_3 Prop and reverse T_3 Prop in 3 separate studies. This 71-year-old woman developed myxedema subsequent to X-ray therapy and total thyroidectomy 4 years before at another institution for Hashimoto's struma. Several months elapsed between each course of treatment because the patient discontinued the medication prescribed; each study thus started from similar levels of myxedema. The response of the B.M.R. and cholesterol during the administration of T_3 and T_3 Prop is shown in FIGURE 5. A comparable decrease in the level of cholesterol was produced by T_3 in doses up to a maximum of 50 μ g., and by T_3 Prop in doses up to 800 μ g. The B.M.R. was unchanged by T_3 Prop during sustained doses of 500 μ g./day. It did not increase with doses of T_3 not exceeding 25 μ g./day. Increase in the B.M.R. occurred when 37 μ g. of T_3 were given. T_3 Prop also increased the B.M.R. to normal when the dose of this analogue was increased to 2 mg./day at a later time not shown on FIGURE 5.

The third study in patient J.E. was made using reverse T_3 Prop. This analogue was administered alone for only 14 days, and then T_3 was given concomitantly in order to test reverse T_3 Prop for antithyroxine properties. FIGURE 6 compares the changes of serum cholesterol during the first 10 to 16 days of each study in this patient. Each time the serum cholesterol decreased about 17 per cent from the pretreatment level, whereas the B.M.R. was unaffected. The decrease of cholesterol after reverse T_3 Prop was accompanied by an increase of creatinuria. Reverse T_3 Prop did not seem to have marked antithyroxine properties at this dose level, since a rise of B.M.R. occurred when T_3 was administered, although not as much as occurred with T_3 alone.

Five other athyreotic patients received T_3 Prop orally for long periods of

time while attending the Thyroid Clinic of the Memorial Center. The relationship of the daily dose of T_3 Prop to the B.M.R. and cholesterol is presented in FIGURE 7. Single points in this graph are averages of several determinations in one patient on a single dose level. The lines connect values determined

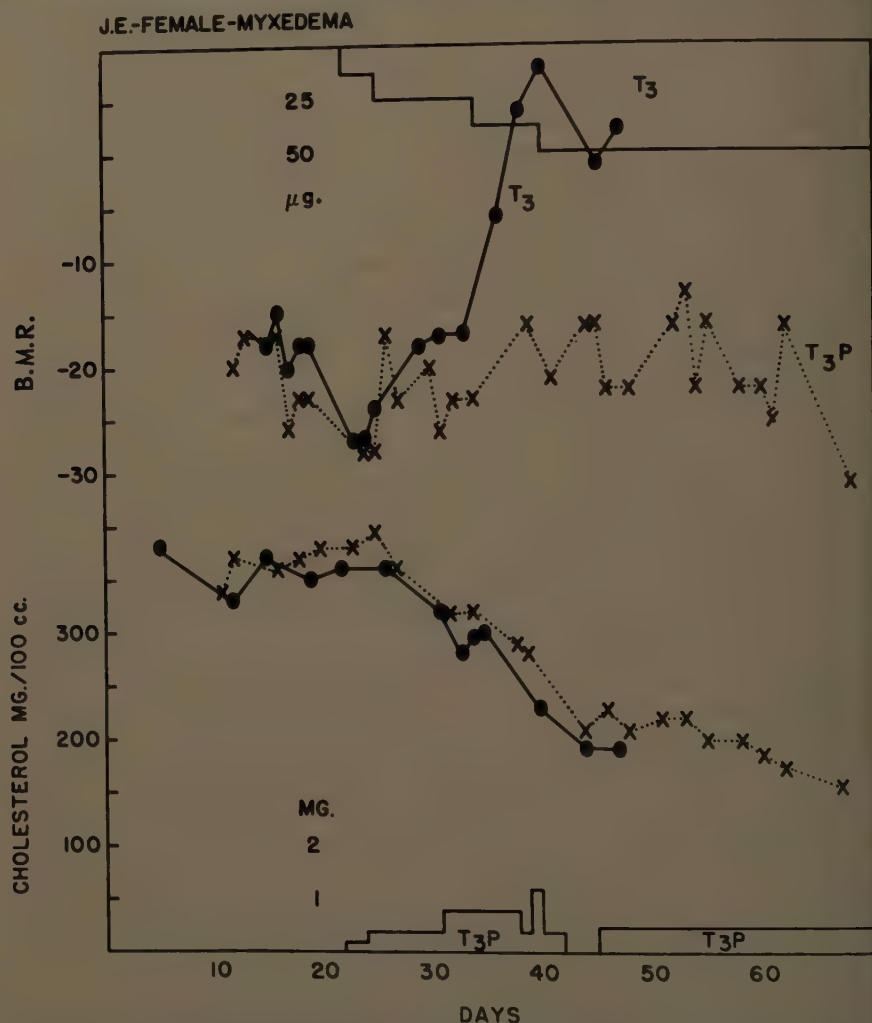


FIGURE 5. The response of the B.M.R. and serum cholesterol during 2 separate studies in patient J.E. Solid lines show data for T_3 ; broken lines show data for T_3 Prop.

for a single patient. The duration of observation ranged from 3 to 10 weeks. Although none of the dose levels shown here exceeded 1.5 mg., it is important to recall that a daily dose of 2.5 mg. will elevate the B.M.R. to normal in the average myxedematous patient.

The effects of T_3 Prop and T_3 on the balances of the nitrogen, phosphorus, calcium, chloride, sodium, and potassium have been compared in patient L.N.,

a 41-year-old woman with myxedema resulting from total thyroidectomy for a thyroid tumor. There was no evidence of recurrence, although the tumor was thought to be malignant when it was removed 2 years before the present study. While the patient received a repetitive, analyzed diet in the hospital, base-line values for the balances, the B.M.R., blood chemistries, and the excretion of

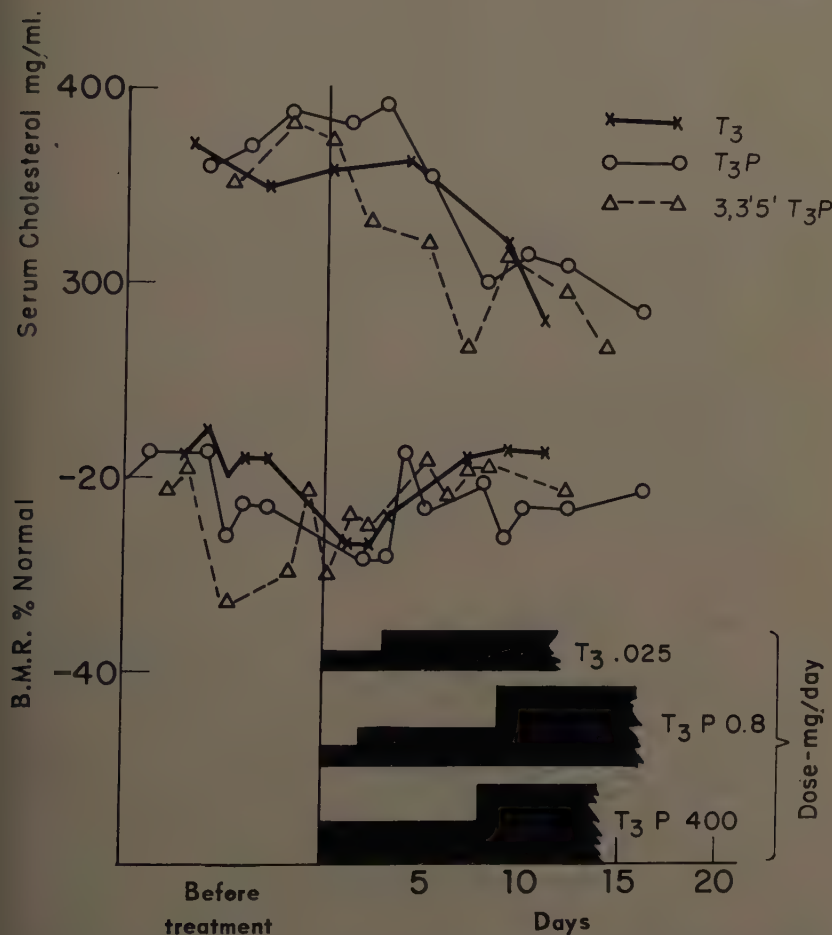


FIGURE 6. Comparison of 2 propionic analogues to triiodothyronine, showing the response of the B.M.R. and serum cholesterol during the initial phases of 3 separate studies in patient J.E.

creatinine and creatinine were established. Ten mg. of T_3 Prop were then given intravenously. The analogue was dissolved in 50 per cent propylene glycol. Chromatographic analysis in *n*-butanol dioxane (80:20, v/v) saturated with 2N NH_4OH ²⁰ and subsequent staining by a modified sulfanilic acid method²¹ showed that the T_3 Prop was contaminated with about 10 to 15 per cent of what was probably tetraiodothyropropionic acid (T_4 Prop) and a smaller amount of an unidentified compound. One week later the patient received

0.5 mg. of L- T_3 intravenously in a similar fashion, except that the drug was dissolved in 0.1 *N* sodium hydroxide.

The effects of these 2 injections on nitrogen and phosphorus balances and on creatine excretion are compared in FIGURE 8. Triiodothyronine, in the dose administered, caused a greater increase in the B.M.R., a larger area under the curve of B.M.R., and a greater increase in urinary nitrogen than was produced by 10 mg. of T_3 Prop. The analogue, however, increased urinary phosphorus and creatine to a greater extent than did T_3 .

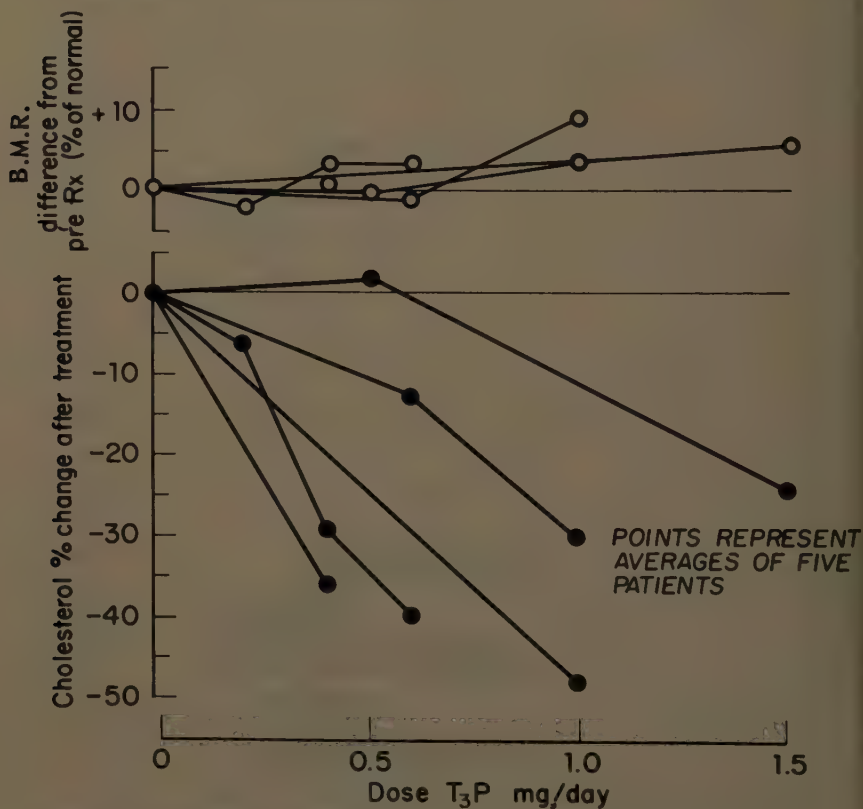


FIGURE 7. The effect of a daily dose of T_3 Prop on B.M.R. and serum cholesterol.

Both T_3 Prop and T_3 caused a negative calcium balance of borderline significance in this patient. Some increase in urinary chlorides occurred in both of the studies. The balances of sodium and potassium were unchanged. Creatinine excretion showed transient and equivocal changes. There were no changes in the levels of blood electrolytes, nitrogen, or glucose, or of serum albumin, globulin, or creatinine.

Review of Findings by Others and Discussion

Distribution and fate. The solubility and stability of the thyroxine analogues have received little attention. Decomposition of some analogues in alkaline

solution²² may invalidate some studies in which these analogues were found inactive. The effect of solubility and route of administration of thyroxine on the potency of thyroxine is well known from the work of Thompson and his collaborators²³⁻²⁷ and, more recently, of Hart and MacLagan.^{28,29} Similar lack

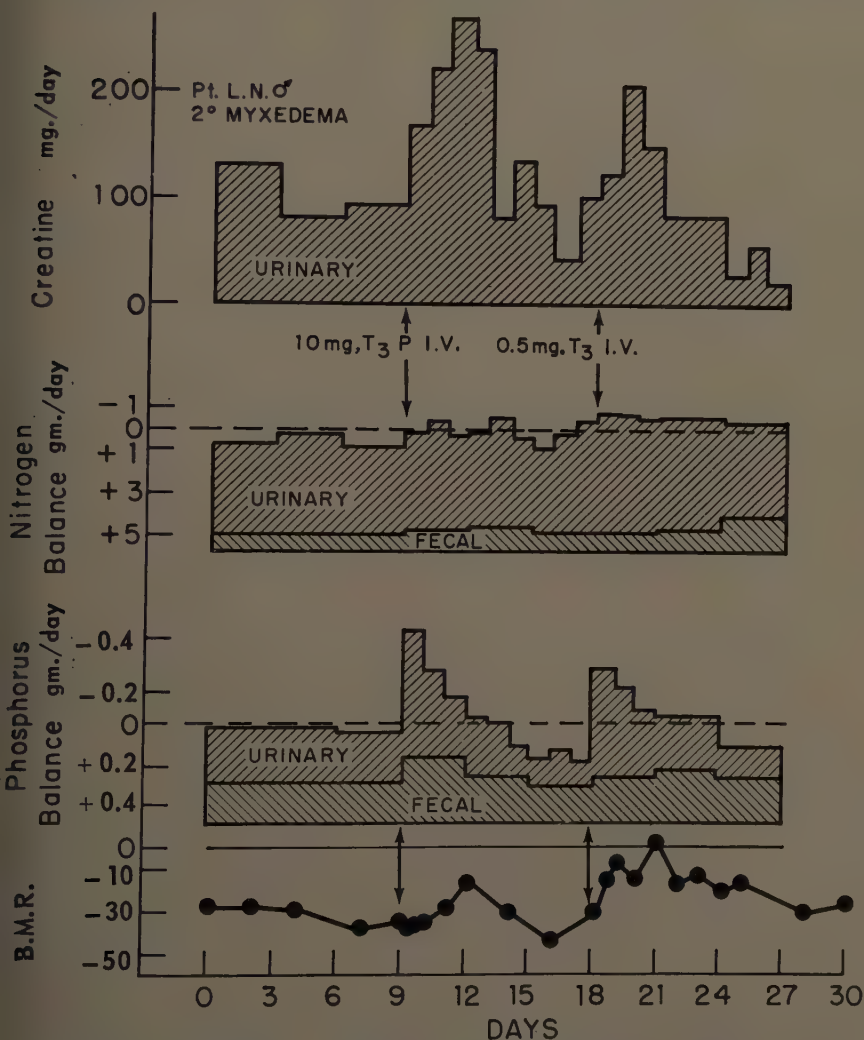


FIGURE 8. A comparison of the effect of T_3 Prop and T_3 on the metabolic balances of nitrogen and phosphorus, on excretion of creatine, and on B.M.R.

of absorption by the oral route may influence the effects of the thyroxine analogues.

Radioiodine labeling of the analogues is an ideal tool for studying their distribution and fate, but only a few analogues have been studied in this manner. Although several labeled thyroxine analogues have been said to be available, the only reports of human studies with labeled analogues found were for

3 others, namely, 3:3'-diiodothyronine,¹³ 3:3':5'-triiodothyronine,¹⁴ and D-thyroxine.³⁰ These analogues and the I³¹-labeled T₃ Prop, reported in this study, disappeared rapidly from the blood in the first hours after injection. With labeled T₃ Prop a slower, final phase of loss from the blood became apparent after the second day. This rate of decrease corresponded to a half time of 2.6 days, quite similar to that reported for labeled T₃.¹⁵

The rate of disappearance from the serum may be modified by protein binding,³¹⁻³⁴ or deiodination, or both. Chromatographic fractionation of the serum and urine show rapid deiodination of labeled 3:3'-diiodothyronine¹³ and of 3:3':5'-triiodothyronine.¹⁴ On the basis of comparison with radioiodide tracers in 2 euthyroid patients reported here, labeled T₃ Prop is also deiodinated. Presuming that radioiodine enters the thyroid only as iodide, deiodination after labeled T₃ Prop must have been rapid, accounting for more than 50 per cent of the injected I³¹ from T₃ Prop in the first day. The rapid deiodination is also reflected by the low percentage of the total iodine constituted by the PBI after T₃ Prop, reverse T₃, and reverse T₃ Prop.

Although rather large doses of the iodinated analogues are required to produce significant changes in the serum PBI, useful information has been obtained from the level of PBI after single intravenous injections or following the last of a series of oral doses of the analogues in athyreotic patients. The iodinated analogues cause elevations of the PBI that are out of proportion to their clinical effects.³⁵⁻³⁷ The rate of decrease of the serum PBI during the interval between 2 and 7 days was more rapid (half time of 2 or 3 days) for T₃,¹⁶ TRIAC,¹⁹ and T₃ Prop than it was for thyroxine.^{15,17,38} The discrepant results concerning the rate of disappearance of reverse T₃ Prop in the 2 studies reported here require further investigation. Reverse T₃ Prop is apparently rapidly deiodinated, and thus might be expected to disappear rapidly from the circulation. The elevation of the total iodine and PBI was much lower after reverse T₃ than after reverse T₃ Prop (FIGURE 4), suggesting that reverse T₃ is poorly absorbed, poorly bound, rapidly excreted, or sequestered somewhere outside the blood stream.

Metabolic effects. Of the facets that constitute the clinical picture of myxedema, such as suborbital fluid collections or mental slowness, almost none has been measured in a quantitative way after the administration of thyroxine analogues. From reports concerning 21 analogues given to myxedematous humans, 14 have relieved the clinical picture when given in sufficient amounts (TABLE 1). Effects on the pulse and body weight are easily quantitated and have generally changed concomitantly with the clinical picture. A few exceptions, such as loss of weight without change in the clinical status or increase in the B.M.R.,⁵⁴ have been reported occasionally.

The B.M.R. has been the most frequent measure of the potency of the thyroxine analogues in human myxedema. Four general types of assay have been applied to the problem. The 2 most widely used assays are based on daily administration, the end point being the determination of the dose required to maintain the B.M.R. near normal in athyreotic patients, or the dose required to elevate the B.M.R. to normal from pretreatment levels during a 2-week interval. This latter assay has been widely applied by Means and Lerman to the various analogues given chiefly via the intravenous route. Two other

methods require the use of single doses, the end point being the measurement of the rise of the oxygen consumption or of the total calories per square meter of body surface produced in excess of those predicted from the pretreatment base line. In each of the 4 methods the weight of sodium L-thyroxine required to produce a similar response may be used as a standard, although some workers have used other compounds as the basis of comparison.

TABLE 2 lists the thyroxine analogues that have been assayed in human myxedema, according to the literature. Our own data, first published in the present communication, are included, but have no reference numbers. Whenever possible, the potencies relative to sodium L-thyroxine have been taken from published reports, but some observers have related potencies to other compounds. In such instances calculation of the potencies relative to thyroxine has been made, using the following assumptions: (1) L-T₃ has 300 per cent of the potency of sodium L-thyroxine, except on the basis of area under

TABLE 1
THYROXINE ANALOGUES REPORTED TO RELIEVE THE CLINICAL PICTURE
OF HUMAN MYXEDEMA

Analogue	References
D-Thyroxine	7, 39-41
N-Formyl DL-thyroxine	28, 29
N-Acetyl L-thyroxine	7, 42
N-Acetyl DL-thyroxyl L-glutamic acid	7, 42
T ₄ Prop	43
TETRAC	35, 43-45
O-Methyl DL-thyroxine	7, 42
Sulfur analogue of thyroxine	7, 42
T ₃ Prop	43
TRIAC	35, 36, 43, 46-49
3:5 DL-diiodothyronine	7, 50
Tetrabromthyronine	7, 51
Tetrachlorthyronine	7, 51
Tribromthyronine	52, 53

the B.M.R. curve, on which potency is equal (*see* TABLE 2); (2) by the oral route DL-thyroxine is 13 per cent and sodium DL-thyroxine is 27 per cent as active as sodium L-thyroxine;^{28, 29} (3) by parenteral routes, DL-thyroxine and sodium DL-thyroxine are about 50 per cent as effective as L-thyroxine or sodium L-thyroxine;³⁹ and (4) when the B.M.R. was unchanged the relative potency is expressed as less than the percentage calculated for the inactive amount of the analogue.

On the basis of the above assumptions, daily oral T₃ Prop had 12 per cent of the potency of sodium L-thyroxine. From the single intravenous doses of T₃ Prop and T₃ given to patient L.N., T₃ Prop had 2 per cent of the activity of thyroxine on the basis of maximum increase in B.M.R. and 0.7 per cent on the basis of total calories produced in excess of those expected from the base line before injection.

In so far as they have been tested, the potencies of the analogues on the B.M.R. in myxedema parallel their potencies on oxygen consumption in rats, summarized elsewhere in this monograph by Money *et al.* No analogue de-

TABLE 2
THE RELATIVE POTENCY BY WEIGHT OF VARIOUS THYROXINE ANALOGUES ON
THE B.M.R. IN HUMAN MYXEDEMA*

Tetraiodinated compounds	† In maintaining a normal B.M.R. (oral)	† In elevating B.M.R. to normal in 2 weeks (I.V.)	† By maximum increase B.M.R. (Single I.V. dose)	† By total excess calories (Single I.V. dose)
Na L-thyroxine (reference com- pound)	100	100	100	100
Na DL-thyroxine	27 (28,29)			
DL-Thyroxine	13 (28,29)	50 (39)		
Na D-thyroxine	2-10 (41)	17 (40)		
	<3 (37)	10 (7) (51)		
		10-12 (39)		
N-Formyl DL-thyroxine	9 (28,29)			
N-Acetyl L-thyroxine		20 (7,42)		4 (25)
N-Acetyl DL-thyroxine				12 (25 S.C.)
N-Acetyl DL-thyroxyl L-glutamic acid		5.5 (7,42)		
Tetraiodothyropropionic acid	2.5 (43)			
Tetraiodothyroacetic acid	3 (43,45)	7 (35)	<25 (36- S.C.)	
O-Methyl Na DL-thyroxine		5.5 (7,35- P.O.)		
Sulfur diphenyl analogue of thy- roxine		12 (42)		
		10 (7)		
Triiodinated compounds				
L-Triiodothyronine (included for reference only)	200-300 (5- I.M., 55) 300-400 (56,57)	400-500 (58)	400 (16)	100 (S.C.)
			200-300 (60S.C., 61)	100 (16,61)
			400-800 (59S.C.)	
D, L-Triiodothyronine	170 (62)		200-400 (54S.C.)	
	150 (55)			
	150-200 (56)			
D-Triiodothyronine			<100 (54- S.C.)	
Triiodothyropropionic acid	5 (43)		2 (38)	0.7
	12			
Triiodothyroacetic acid	4 (46)	17-25 (35)	38-45 (36- S.C.)	12 (36S.C.)
	5 (43)		20 (19,46- P.O.)	
	5-8 (49)			
	<10 (47- I.V.)			
	>8 (48)			
Triiodothyroformic acid	<1.5 (35- I.V.)			
3:3':5' DL-Triiodothyronine	<0.7 (63)			
3:3':5' Triiodothyropropionic acid	<0.07			
Diiodinated compounds				
3:5 DL-Diiodothyronine	2 (50)	1.3 (7)	3 (25)	0.2 (25)
3':5' DL-Diiodothyronine	<25 (64)			
2':6' DL-Diiodothyronine	<0.6 (42- I.V.)			
Noniodinated compounds				
Thyronine		0 (7)	<0.25 (25)	
Tetrabrom DL-thyronine		6 (51)		
		3 (7)		
Tetrachlor DL-thyronine		0.6 (51)		
		0.2 (7)		
3:5:3' Tribrom DL-thyronine	40 (52 I.M.)	40 (53)		

* Numbers in parentheses are reference numbers.

† Exceptions to the routes of administration shown at the top of the columns are noted.

scribed is more potent than the 2 thyroid hormones in this regard. For the acetic acid and propionic acid analogues, the 3:5:3'-triiodinated forms have at least as much and probably more effect on the B.M.R. than the tetraiodinated forms. The greater effect of T_3 in comparison to thyroxine is widely accepted, of course, for 3 of the 4 types of change in the B.M.R., as shown in TABLE 2. Although it has been suggested that the difference in potency of T_3 and thyroxine may be influenced by binding in the blood,³⁴ the greater potency of T_3 when administered over long periods of time probably negates this theory. There does not seem to be any correlation between potency and the rate of loss from the blood among the thyroxine analogues.

The triiodinated analogues, especially TRIAC,^{49, 65-67} have a more rapid effect on B.M.R. than does either thyroxine or TETRAC.⁴⁵ It is widely agreed that T_3 changes the B.M.R. sooner than thyroxine,^{16, 49, 57, 59, 61, 65, 67} although Kyle and his co-workers⁶⁰ dissent. From the literature it is difficult to decide whether TRIAC produces significant increases of B.M.R. prior to 24 hours,^{49, 65, 67} or later.^{19, 60, 68} T_3 Prop did not produce a change in the B.M.R. as rapidly as did T_3 in the patient reported here, but the dose of T_3 was relatively larger than that of T_3 Prop by maximum increase in the B.M.R. and by area under the B.M.R. curve. An earlier onset may have been due to a greater effect from the dose used, rather than to any other difference. T_3 apparently may affect the B.M.R. several hours after administration.^{16, 59} The duration of the elevation of B.M.R. produced by a single dose or after stopping multiple doses of the 3:5:3'-triiodinated analogues is less^{19, 36, 38, 49} than the duration produced by thyroxine. The same may be said for T_3 .^{16, 49, 61} The prolonged effect of large doses of TRIAC on the B.M.R. of certain goitrous cretins, reported by Zondek and his co-workers,^{66, 69, 70} may be an exception.

Previous workers, when attempting to correlate structure and function of the analogues, have specified that the compounds should relieve all the known alterations of myxedema.^{6, 7, 71} By applying this restriction they have hoped to avoid taking into account nonspecific effects in myxedema not mediated through pathways analogous to thyroxine.⁸ Such restrictions may be conceived as eliminating dinitrophenol-like elevations of B.M.R.⁷²⁻⁷⁴ or salicylate-like elevations of B.M.R. and decreases in cholesterol.⁷⁵⁻⁷⁷ Calling an effect nonspecific does not add to our understanding of mechanisms, however, and may exclude from study compounds active only when given in relatively large amounts.

Most observers now agree with Lerman and Pitt-Rivers,⁷⁸ who first described it, that TRIAC in small doses can reduce the serum cholesterol in human myxedema without concomitant increase in B.M.R. The evidence concerning separation of these 2 effects is summarized in TABLE 3 for TRIAC, other analogues, and hormones. Hill and his co-workers⁴³ have also found the separation with T_3 Prop, and believe it occurs with T_3 , T_4 Prop and TETRAC. In patient J.E., reverse T_3 Prop produced a separation but, since this analogue was only studied once, repetition will be necessary. The failure of some workers to observe a separation may have been due to the use of single doses, to large initial doses, or to failure to continue observations for a sufficient length of time. In our experience each of these factors may operate to obscure the separation. Even under optimal conditions, however, the separation has not been demonstrable

in all patients. TRIAC and T₃ Prop, of the compounds tested, are most likely to produce separation, but it occurs with other analogues and with the thyroid hormones. The separation is a dose effect, since the compounds causing it will elevate the B.M.R. to normal if given in sufficient dosage.

Since this type of separation of effect is dependent on the administration of doses too low to elevate the B.M.R., it is probably due to differences in the threshold. Such differences in threshold, or X-intercept dosage, have been described for TETRAC, T₃ Prop, and T₄ Prop by Stasilli *et al.*²² for antigoitrogenesis and oxygen consumption in rats. The usual method of expressing potency of analogues in terms of percentage of L-thyroxine does not contain

TABLE 3

THYROID HORMONES AND ANALOGUES THAT REDUCE SERUM CHOLESTEROL WITHOUT AN ACCOMPANYING INCREASE IN B.M.R.

Compound	Dose (mg.)	Route	% Reduction of cholesterol	Days of observation	Patients with reduction	Reference
					without reduction	
Na L-thyroxine	6	S.C.*	20	6	1/2	59
DL-Thyroxine	0.3-1.2	oral	50	28	1/8	29
L-Triiodothyronine	0.003-0.012	oral	40	39	2/0	49
Tetraiodothyroacetic acid	10	oral	30-50	16	2/0	45
Triiodothyroacetic acid	1-4	I.V.	20-50	16-17	2/0	78
	<1.5	I.V.	22-50	10-17	4/1	35
	1	I.V.	30	13	1/0	47
	0.5-1.0	oral	43	6	2/1	49
	0.5-1.0	oral	14	35	1/0	79
	2-4	oral	40	7	2/0	46
	4	oral	—	5	1/1	80
	2	oral	—	—	1/0	45
	18	oral*	23	3	1/1	67
Triiodothyropropionic acid	0.4-1.0	oral	50	45	1/0	38
	0.2-1.5	oral	30 (average)	45	5/0	38

* Indicates single dose; all others administered daily.

information about threshold, and thus can not be used to describe such separations.

The fact that thyroxine causes an increase in urinary nitrogen in myxedematous patients maintained on a constant diet⁸¹ was known before the structure of thyroxine was elucidated.² Similar increases in nitrogen loss have been reported following single doses of T₃,^{16,36,59,60,82} TRIAC,^{19,36,47,82} TETRAC,¹⁹ and T₃ Prop. T₃ produces a change in nitrogen balance more quickly than does thyroxine.^{16,59} In one patient with myxedema TRIAC was reported by Graeff and his co-workers⁴⁷ to cause azoturia and decreased serum cholesterol without increasing the B.M.R.

Phosphaturia is also increased by thyroxine,^{16,59,83} T₃,^{16,19,36,38,49,59,82} TRIAC,^{19,36,47,49,82} and T₃ Prop.³⁸ In the patient described by Graeff and his co-workers⁴⁷ both nitrogen and phosphorus loss were increased without change in the B.M.R. Asper⁵⁴ found that 1 mg. of D-T₃ caused a loss of phosphorus and nitrogen without effect on the B.M.R. or the clinical state of the patient.

In patient L.N., described here, the phosphaturia induced by T_3 Prop was out of proportion to the azoturia and the fall in serum cholesterol. In this same patient such a marked disproportion was not produced by T_3 .

Marked increases in urinary creatine have been reported in myxedematous patients by others who were administered thyroxine,^{16,81} T_3 ,^{16,19} TRIAC,^{19,36,47,49,82} and TETRAC.^{19,35} In patient L.N. the curve of urinary creatine was more like that for urinary phosphorus than that for urinary nitrogen or the B.M.R. In patient J.E. daily administration of reverse T_3 Prop produced increased creatinuria accompanying a fall in serum cholesterol, but without an increase of B.M.R. Graeff and his co-workers⁴⁷ obtained a similar result with TRIAC.

The effect of thyroid hormones and analogues on calcium balance is in dispute. Aub and his coworkers⁸³ found that the excretion of calcium was 40 per cent below normal in myxedema, and the excretion was increased by the daily administration of desiccated thyroid, despite continued low calcium intake. The administration of a single dose of 10 mg. of DL-thyroxine caused no significant change in calcium balance 4 days later in 1 myxedematous patient. Negative balance occurred in 1 of 2 other patients who were observed for several weeks after receiving 20 to 30 mg. of thyroxine. Significant losses of nitrogen and phosphorus were produced by these doses of thyroxine, however. Negative calcium balance was consistently obtained in Aub's patients only when desiccated thyroid was given daily. This effect of daily doses of desiccated thyroid has been confirmed by Robertson.⁸⁴ It seems likely that single doses of thyroactive substances will produce significant losses of calcium in only an occasional myxedematous patient, but that chronic therapy is more effective in this regard. Single doses of 3 mg. of sodium L-thyroxine intravenously¹⁶ or 4.8 mg. subcutaneously⁵⁹ had no effect on calcium balance. One mg. of T_3 intravenously caused a negative calcium balance of equivocal significance in 2 myxedematous patients,^{16,19} and no effect when given subcutaneously in a different study.⁵⁹ TRIAC and TETRAC caused no change in urinary calcium, but TETRAC increased stool calcium and thus caused a negative balance.¹⁹ In the present study, T_3 Prop had a result similar to that with TETRAC. Because of the relief of constipation, such changes in stool output must be interpreted with caution. It appears possible that there is a higher threshold for the effect of thyroid hormones and analogues on calcium balance than on nitrogen or phosphorus balance.

Sodium, potassium, and chloride balances in myxedematous patients are only irregularly affected by the thyroid analogues and hormones. Thyroxine did not alter chloride balance in 2 acute studies,^{16,81} whereas T_3 , TRIAC, TETRAC, and T_3 Prop^{19,38} caused increased urinary chloride. Byrom⁸⁵ reported losses of both sodium and potassium following the administration of large doses of DL-thyroxine to myxedematous patients. Doses of L-thyroxine and T_3 , judged somewhat smaller in terms of expected effect on the B.M.R., produced no effect on the excretion of either cation.^{16,19,59} Ten mg. of T_3 Prop also had no effect. Negative sodium balance without loss of potassium has been reported from 15 mg. of TRIAC and 20 mg. of TETRAC.¹⁹

In the serum, several lipid fractions, in addition to cholesterol which was discussed above, have been reported to decrease after the administration of

thyroid hormones or analogues.^{48, 63, 79} There have been no reports of significant changes in the levels of blood electrolytes, nitrogen, or glucose, or of serum albumin, globulin, or creatinine from the use of the hormones or their analogues.

Summary

Some studies of the distribution and fate of 3:5:3'-triiodothyropropionic acid have been reported. This analogue disappeared from the circulation and was deiodinated at a rapid rate. A comparison of the distribution and fate of this analogue with other analogues and hormones administered to euthyroid and myxedematous patients has been made by means of a review of the literature.

It was found that 3:5:3'-triiodothyropropionic acid had 12 per cent of the potency of sodium L-thyroxine in maintaining the B.M.R. at normal levels in 10 myxedematous subjects. The use of 3:3':5'-triiodothyropropionic acid had no significant effect on B.M.R. in daily oral doses up to 400 mg. in a 14-day study of one myxedematous patient. A comparison of these B.M.R. effects with those reported for other analogues has been made.

A decrease in serum cholesterol without an accompanying increase in B.M.R. occurred when 3:5:3'-triiodothyropropionic acid was given to myxedematous patients. This separation of effect was seen with small, daily doses and disappeared when the dosage was increased. The separation has been compared with similar observations during the administration of TRIAC, TETRAC, and the thyroid hormones, and its significance has been discussed.

During metabolic balance studies in a myxedematous patient, 10 mg. of 3:5:3'-triiodothyropropionic acid caused a marked phosphaturia and creatinuria, accompanied by a less marked azoturia and increase in the B.M.R. As compared with the results of a subsequent study in the same patient with 0.5 mg. of 3:5:3'-triiodothyronine, 3:5:3'-triiodothyropropionic acid produced increases in urinary phosphorus and creatine which were out of proportion to its effect on B.M.R. and nitrogen excretion. The literature pertaining to the effects of thyroxine analogues on metabolic balance in human myxedema has been reviewed.

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THE METABOLIC EFFECTS OF THYROID ANALOGUES IN PSYCHIATRIC PATIENTS

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Introduction

The purpose of our current investigations is the exploration of possible relationships between alterations in thyroid function and psychiatric disorders. Emotional and psychopathological changes are known to occur in patients with hyperthyroidism or myxedema.^{1,2} Hostility and lability of emotions are common concomitants of thyroid overactivity. Myxedema may be associated with retardation of mental processes, apathy, depression of mood, paranoid misinterpretations, delusions, and hallucinations. Deviations in the indices of thyroid function among schizophrenic patients have been reported by various investigators. Bowman³ and Hoskins⁴ found lowered basal metabolic rates in a large number of schizophrenic patients and described unusually high tolerance among these patients to the administration of large doses of desiccated thyroid. Brody and Man⁵ reported normal serum protein-bound iodine levels in similar patients. Cranswick⁶ described abnormally elevated thyroïdal uptakes of I^{131} in a majority of schizophrenic patients. Reiss⁷ reported that in a mixed diagnostic group of psychiatric patients the thyroïdal uptake of I^{131} was often unusually high or low, returning to normal ranges only after the patients had responded favorably to various therapies.

A review of such studies suggested to us the need to clarify the following considerations: (1) whether the administration of thyroid hormones to psychiatric patients might be associated with well-defined emotional or psychopathological changes; (2) whether variations in the indices of thyroid function could be detected in psychiatric patients studied under rigorous control conditions; and (3) whether thyroid hormones, once produced, are adequately metabolized and utilized in certain psychopathological states.

We have previously reported that the administration of 3:5:3'-triiodothyronine to certain euthyroid patients in various psychopathological states was associated with the appearance of formerly hidden or absent hostile emotions and sexual unrest. In many of these patients a marked decrease in apathy, depression of mood, and depersonalization was observed. In other patients, however, the administration of triiodothyronine was not associated with any significant emotional changes.^{8,9}

In order to clarify the metabolic effects of triiodothyronine in the setting of various psychopathological states, 13 patients were investigated on the Metabolic Unit of the Payne Whitney Clinic. The results of this study have been reported in detail elsewhere,¹⁰ but it seems desirable to review the pertinent

observations here. These 13 patients were maintained on constant diets. Complete urine and stool collections were made. Following control periods each patient received triiodothyronine in daily oral doses of 100 to 300 μ g. for 2 to 3 weeks. Seven patients in this group responded to hormone administration by significantly increasing fecal calcium excretion, thereby establishing negative calcium balances. Six patients demonstrated, while receiving triiodothyronine, either no change in calcium balance or a significant retention of calcium, primarily due to a decrease in fecal calcium loss. In general, those patients who developed negative calcium balances while receiving triiodothyronine also demonstrated strong emotions during hormone administration; the mean duration of illness in this group was 12 months. Those patients who failed to develop a negative calcium balance during the experiment also failed to demonstrate any significant emotional changes while receiving triiodothyronine; moreover, the mean duration of illness of this group was 6 years, and apathy, withdrawal, and paranoid features were prominent findings.

Since the publication of this study 3 more schizophrenic patients, whose illnesses had each been manifest for more than 15 years, were investigated in a similar manner. None revealed any significant increase in fecal calcium excretion during triiodothyronine administration, and none showed any significant emotional changes during the procedure.

An important relationship between brain function and thyroid metabolism was established by Tata,¹¹ who demonstrated that cerebral cortical tissue was capable of metabolizing labeled thyroxine to 3:5:3'-triiodothyronine and to tetraiodothyropropionic acid and/or tetraiodothyroacetic acid, followed by deiodination to triiodothyropropionic acid and/or triiodothyroacetic acid and, finally, to iodide. Rawson and his associates¹² studied the effect of triiodothyronine on nitrogen and electrolyte metabolism in patients with myxedema, and they reported an immediate and significant increase in urinary nitrogen and phosphorus excretion following the intravenous administration of 1000 μ g. of this hormone. No increase in urinary calcium was observed.

In order to clarify the meaning of our observations of the effects of triiodothyronine administration on calcium balance in psychiatric patients, it seemed desirable to determine the influence of intravenously administered triiodothyronine on urinary nitrogen, calcium, and phosphorus excretion, and to compare this effect with that produced in psychiatric patients by the intravenous administration of triiodothyropropionic acid.

Method

Ten selected patients were placed on the Metabolic Unit of the Payne Whitney Clinic. Nine were diagnosed as having schizophrenic reactions, although the specific psychopathological features and the duration of illness varied widely from patient to patient; the tenth was diagnosed as having a personality disorder (psychopathic personality) with chronic alcoholism. All patients were free of any known physical disorders, particularly endocrine dysfunctions. Dietary intakes were constant throughout the entire period of study. Urines were collected and analyzed for nitrogen, calcium, and phosphorus content according to accepted methods.¹² Urinary creatine and creatinine determinations were made, the latter to assure accuracy of urine collections.

Following control periods 6 of these patients received 500 to 1000 $\mu\text{g.}$ of triiodothyronine intravenously on 1 or 2 occasions. Three patients received triiodothyronine similarly, preceded or followed by the intravenous administration of 25 mg. of triiodothyropropionic acid. In one patient, only triiodothyropropionic acid was given.

Continuing observations of the emotional and psychopathological states of these patients were carried out by the research psychiatrists, individual psychotherapists, and the psychiatric nursing staff on the unit. The special behavior charts described by Kohl¹³ were also employed for this purpose. Definitions of special terms, such as hostility or sexual unrest, may be found in a previous publication.¹⁰

Five patients in this series also received triiodothyronine in daily oral doses of 100 to 200 $\mu\text{g.}$ for 2 to 3 weeks, while nitrogen, calcium, and phosphorus balance determinations were performed.

Observations

The administration of a single, intravenous dose of triiodothyronine or triiodothyropropionic acid was seldom associated with any significant degree of emotional or psychopathological change. One notable exception was a 20-year-old woman (patient C), who was diagnosed as having a schizophrenic reaction of the catatonic type, and who demonstrated marked tension, anxiety, hostility, and sexual unrest, with a concomitant decrease in depression of mood and withdrawal following her first intravenous injection of 500 $\mu\text{g.}$ of triiodothyronine. However, several patients who failed to show emotional changes during this procedure subsequently manifested intense hostility or sexual unrest when triiodothyronine was given continuously in daily oral doses of 100 to 200 $\mu\text{g.}$

Interesting variations were noted in the metabolic responses of these patients to the intravenous administration of these thyroid analogues. These are described in detail below.

Patient A (PWC-2). This 33-year-old man was diagnosed as having a personality disorder (psychopathic personality) with chronic alcoholism of 15 years' duration. At the time of study he presented no overt psychopathological symptoms. His basal metabolic rate (BMR) was -15 per cent. The serum protein-bound iodine (PBI) level was 7.3 $\mu\text{g./100 ml.}$ His constant daily diet contained 14.95 gm. nitrogen, 1.408 gm. calcium, and 1.511 gm. phosphorus. During the control period his average daily urinary nitrogen excretion was 11.27 gm., urinary calcium 0.268 gm., and urinary phosphorus 1.150 gm.

On the sixteenth day of this study 500 $\mu\text{g.}$ of triiodothyronine was given intravenously. Because of technical difficulties it became necessary to discard the urine collections for the next 72 hours; hence the metabolic response to this procedure could not be evaluated. At the end of the second control period of 8 days, another 500- $\mu\text{g.}$ dose of triiodothyronine was administered intravenously. The metabolic response is shown in FIGURE 1. Within 24 hours urinary nitrogen excretion rose to 14.48 gm. and urinary calcium to 0.304 gm., and subsequently reached a peak of 0.324 gm., while urinary phosphorus rose to 1.531 gm. The elevation in nitrogen excretion lasted for 6

days. The elevations of urinary calcium and phosphorus each lasted for 3 days.

This patient therefore responded to triiodothyronine administration with a prompt increase in urinary nitrogen, calcium, and phosphorus excretion. No significant emotional changes were observed during this study.

Patient B (PWC-5). This 22-year-old woman was diagnosed as having a schizophrenic reaction of undifferentiated type. She presented an illness of

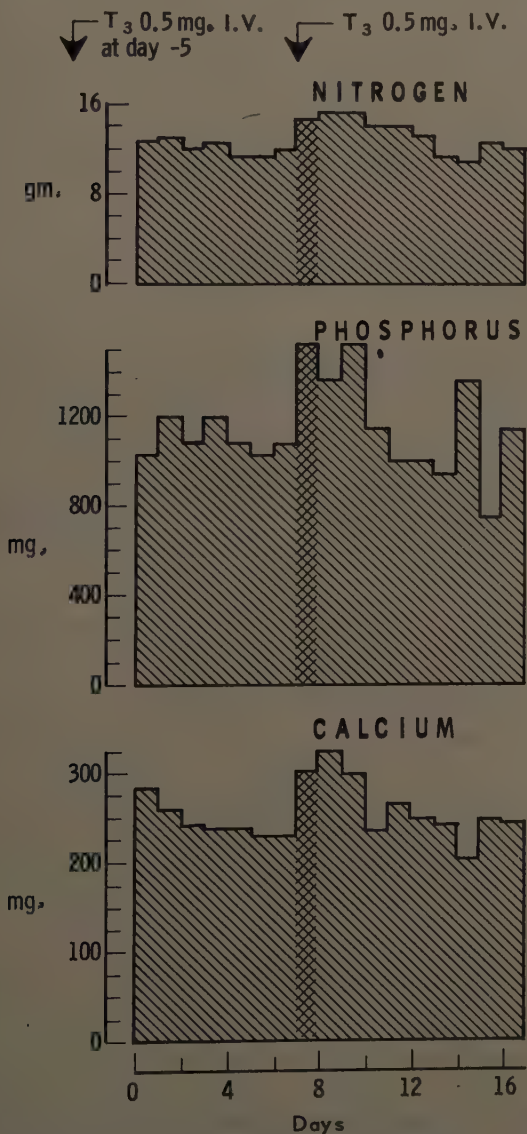


FIGURE 1. The metabolic effects of intravenous triiodothyronine in patient A, a 33-year-old male with a personality disorder and chronic alcoholism.

4 months' duration, with many prominent psychoneurotic symptoms, in particular recurrent anxiety attacks, concentration difficulty, hypochondriacal ruminations, loss of energy, feelings of unreality, and loss of sexual interest.

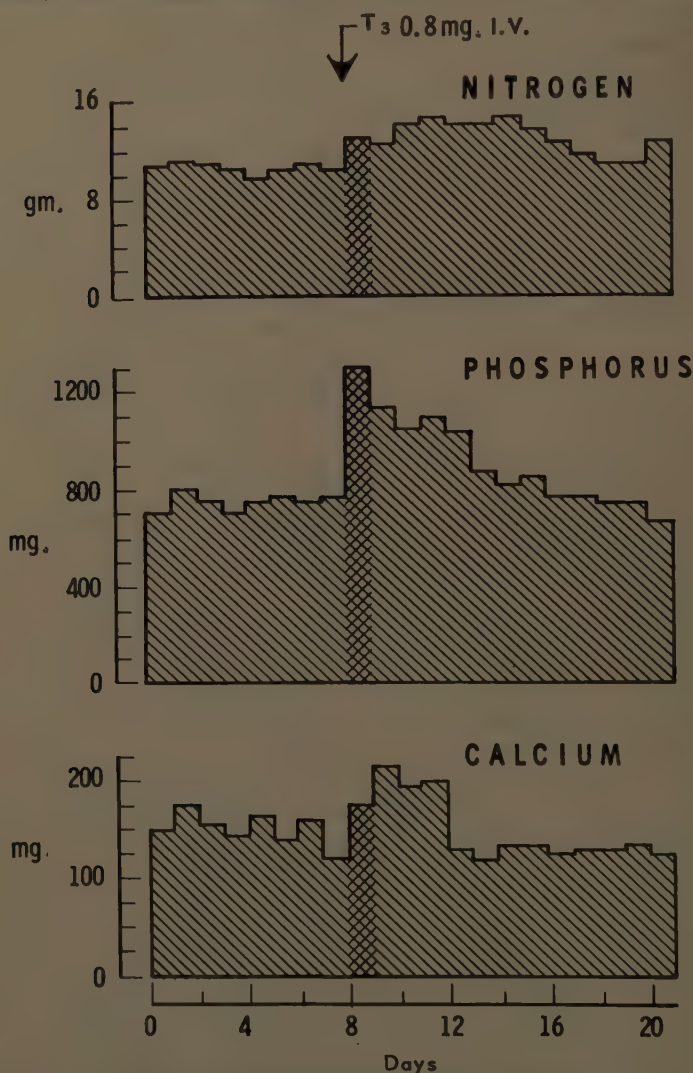


FIGURE 2. The metabolic effects of intravenous triiodothyronine in patient B, a 22-year-old female with an early, undifferentiated schizophrenic reaction.

Her BMR ranged from -12 to -22 per cent. The PBI was $6.3 \mu\text{g.}/100 \text{ ml.}$ The thyroidal uptake of I^{131} was 37 per cent with a conversion ratio of 29 per cent. Her constant daily intake contained 11.91 gm. of nitrogen, 0.809 gm. of calcium, and 1.217 gm. of phosphorus. During the control period daily urinary nitrogen excretion averaged 10.70 gm., urinary calcium 0.157 gm., and urinary phosphorus 0.756 gm.

On the fifteenth day of study, 800 μ g. of triiodothyronine was administered intravenously. The metabolic effects are shown in FIGURE 2. Urinary nitrogen excretion increased promptly to 13.05 gm., remaining elevated for 10 days. Urinary calcium excretion increased promptly to 0.175 gm., subsequently reaching a peak of 0.214 gm. and remaining elevated for 5 days. Urinary phosphorus excretion increased to 1.295 gm., remaining elevated for 9 days. Thus, the metabolic effects of triiodothyronine in this patient were characterized by an immediate increase in urinary nitrogen and calcium and phosphorus excretion following hormone administration.

Following the intravenous dose of triiodothyronine this patient demonstrated a moderate degree of sexual unrest. Subsequently this patient received triiodothyronine in daily oral doses of 200 to 300 μ g. for 3 weeks. At that time sexual unrest increased in intensity, and hostile emotions appeared

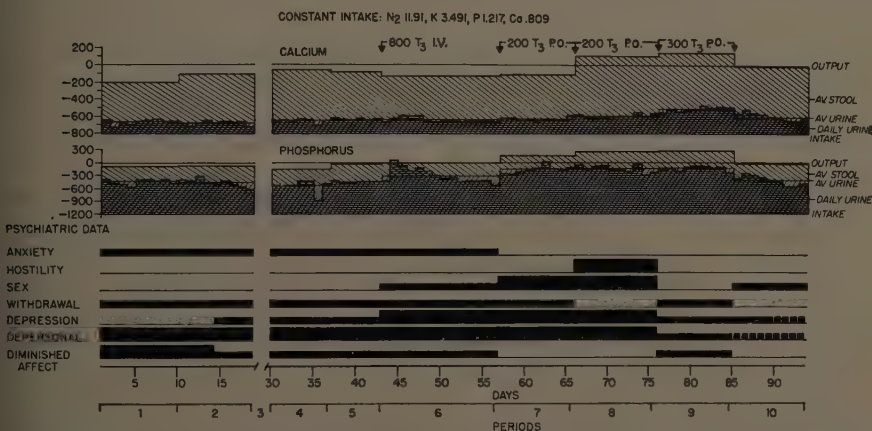


FIGURE 3. The metabolic and clinical effects of triiodothyronine in patient B, a 22-year-old woman with an early undifferentiated schizophrenic reaction. Reproduced with permission from *The Journal of Clinical Endocrinology and Metabolism*.¹⁰

for the first time. Following termination of triiodothyronine, these emotions subsided, and the patient began to improve gradually and steadily. Metabolic balance studies carried out during this experiment are shown in FIGURE 3.¹⁰ The sustained administration of oral triiodothyronine was associated with the induction of a negative calcium balance, resulting largely from an increase in fecal calcium excretion.

Patient C (PWC-4). This 20-year-old woman was diagnosed as having a schizophrenic reaction of the catatonic type. She presented a 6-month history of withdrawal, depression of mood, loss of interest in her surroundings, unreality feelings, and marked diminution in overt manifestations of emotions. Her BMR was initially depressed, ranging from -28 to -35 per cent, but no clinical evidence of hypothyroidism was present. Her PBI was 5.2 μ g./100 ml. She was maintained on a constant daily intake containing 13.10 gm. nitrogen, 0.869 gm. calcium, and 2.227 gm. phosphorus. During the control period daily urinary nitrogen excretion averaged 11.90 gm., urinary calcium 0.097 gm., and urinary phosphorus 0.757 gm.

On the thirteenth day of study 500 μ g. of triiodothyronine was administered intravenously. Urinary nitrogen excretion rose promptly to 14.85 gm., remaining elevated for 3 days. Within 24 hours urinary calcium increased to 0.127 gm., reaching a peak of 0.150 gm. and remaining elevated for 3 days. Urinary phosphorus increased immediately to 1.235 gm., remaining elevated for 3 days.

Seven days after the first injection, a second dose of 500 μ g. of triiodothyronine was administered intravenously. After a 24-hour delay urinary nitrogen increased to 18.80 gm., remaining elevated for 3 days. After a 24-hour delay urinary calcium increased to 0.193 gm., the elevation lasting for 2 days. Within the first 24 hours urinary phosphorus increased to 1.010, remaining elevated for 3 days.

This patient demonstrated a prompt rise in urinary nitrogen, calcium, and phosphorus following the first injection of triiodothyronine, but a 24-hour delay before the increase in nitrogen or calcium appeared following the second dose of triiodothyronine. The patient manifested marked tension, anxiety, anger, feelings of hopelessness, and sexual unrest immediately after the first dose of triiodothyronine. This state of strong emotions lasted for 4 days and was followed by a transient period of relative well-being. The second intravenous dose of triiodothyronine failed to produce a similar change in her emotional state. Subsequently she was placed on triiodothyronine in daily oral doses of 100 to 200 μ g. for 2 weeks. During the period of hormone administration intense anxiety, hostility, and sexual unrest were prominent. Following its termination, the patient returned to her original psychopathological state of depression, withdrawal, and mutism for 6 days, whereupon an abrupt and dramatic clearing of her psychopathological condition occurred. Balance determinations were done throughout the period of study, and a markedly negative calcium balance was established during the period of oral administration of triiodothyronine; this change was due primarily to an increase in fecal calcium excretion.¹⁰

Patient D (PWC-8). This 26-year-old woman was diagnosed as having a schizophrenic reaction of the undifferentiated type. She presented a 6-year history of anxiety, depression of mood, concentration difficulty, and intermittent episodes of depersonalization. Her BMR ranged from plus 3 to plus 12 per cent. Her PBI was 5.7 μ g. per 100 ml. She was maintained on a constant daily intake containing 15.60 gm. nitrogen, 1.104 gm. calcium, and 1.513 gm. phosphorus. During the control period daily urinary nitrogen excretion averaged 13.92 gm., urinary calcium 0.223 gm., and urinary phosphorus 0.969 gm.

On the fifteenth day of study, 500 μ g. of triiodothyronine was administered intravenously. Within 24 hours a prompt rise in urinary nitrogen to 16.15 gm. was observed, and this elevation continued for 6 days. Within 24 hours, urinary calcium increased to 0.265 gm., reaching a peak of 0.317 gm. and remaining elevated for 3 days. Urinary phosphorus increased promptly to 1.540 gm., remaining elevated for 3 days.

Twelve days after the first injection another 500- μ g. dose of triiodothyronine was administered intravenously. This time a delay of 24 hours occurred before urinary nitrogen increased to 17.71 gm. It remained elevated for five

days. After a 24-hour delay, urinary calcium increased to 0.301 gm., remaining elevated for 2 days. Within 24 hours, urinary phosphorus rose to 1.361 gm., and remained elevated for 4 days.

This patient therefore demonstrated a prompt increase in urinary nitrogen, calcium, and phosphorus following the first injection of triiodothyronine, but a delay of 24 hours before any change in urinary nitrogen or calcium was seen after the second dose of the hormone. This patient demonstrated a moderate degree of sexual unrest and hostility following triiodothyronine administration.

Patient E (PWC-7). This 40-year-old premenopausal woman was diagnosed as having a schizophrenic reaction, paranoid type, of more than 20 years' duration. For 6 months prior to study severe depression of mood, paranoid delusions, withdrawal, and depersonalization characterized her condition. At the time of study she was resentful and suspicious, demonstrating little spontaneity or interest in her surroundings. Her BMR was -19 per cent, and her PBI was 5.5 μ g./100 ml. She was maintained on a constant daily intake containing 12.80 gm. of nitrogen, 1.303 gm. of calcium, and 1.262 gm. of phosphorus. During the control period daily urinary nitrogen excretion averaged 10.99 gm., urinary calcium 0.270 gm., and urinary phosphorus 0.759 gm.

On the ninth day of study she received 500 μ g. of triiodothyronine intravenously; the metabolic findings are shown in FIGURE 4. No effect on urinary nitrogen could be observed. Urinary calcium excretion rose to 0.358 gm. within 24 hours, reaching a peak of 0.378 gm. and remaining elevated for 4 days. Within 24 hours urinary phosphorus rose to 1.149 gm., remaining elevated for 4 days.

Eight days later a second injection of 500 μ g. of triiodothyronine was administered. Again, no change in urinary nitrogen was seen. A delay of 24 hours occurred before an increase in urinary calcium excretion to 0.350 gm. was noted; this elevation continued for 3 days. After a delay of 24 hours, urinary phosphorus rose slightly to 0.940 gm., this increase lasting for only a day.

This patient therefore failed to demonstrate an increase in urinary nitrogen excretion following triiodothyronine administration on 2 occasions. Although urinary calcium and phosphorus increased promptly following the first injection of the hormone, a delay of 24 hours was noted before any effect was seen following the second injection. Moreover, the increase in urinary phosphorus at this time was markedly limited in duration and degree.

In association with the administration of triiodothyronine, overt manifestations of paranoid misinterpretations, resentment, depersonalization, and withdrawal diminished, although depression of mood remained essentially unaffected. Evidence of slight sexual unrest was noted. Subsequently this patient received triiodothyronine in daily oral doses of 100 to 200 μ g. for 2 weeks. Balance determinations were carried out. In contrast to the previous cases presented, this patient revealed a marked increase in calcium retention during the oral administration of triiodothyronine. These findings are shown in FIGURE 5.¹⁰

Patient F (PWC-9). This 39-year-old woman was diagnosed as having a schizophrenic reaction, hebephrenic type, of 15 years' duration. At the time of our study she was apathetic, withdrawn, and uncommunicative. Her

BMR was -23 per cent. The thyroïdal uptake of I^{131} was 26 per cent, with a conversion ratio of 23 per cent. Her constant daily intake contained 12.45 gm. nitrogen, 0.802 gm. calcium, and 1.201 gm. phosphorus. During the control period daily urinary nitrogen excretion averaged 11.02 gm., urinary calcium 0.144 gm., and urinary phosphorus 0.712 gm.

On the nineteenth day of this study 1000 μ g. triiodothyronine was administered intravenously. No emotional changes were observed. Within 24

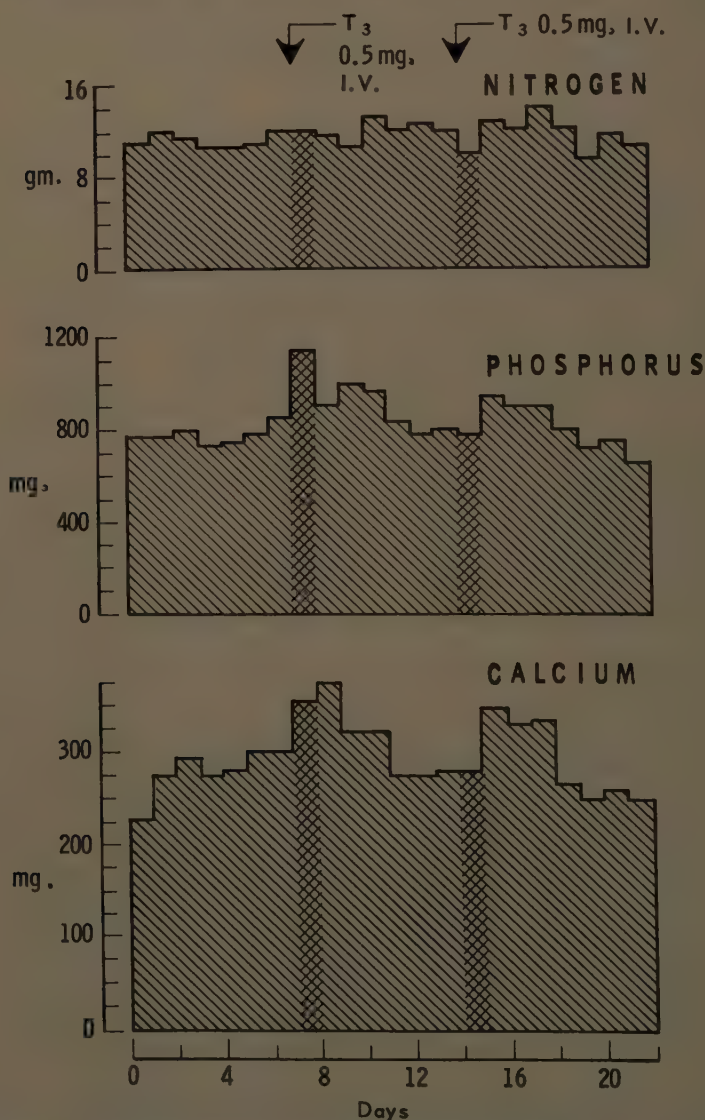


FIGURE 4. The metabolic effects of intravenous triiodothyronine in patient E, a 40-year-old female with a chronic paranoid schizophrenic reaction.

hours urinary nitrogen excretion rose to 14.14 gm., and the elevation continued for 6 days. No significant increase in urinary calcium excretion was noted.

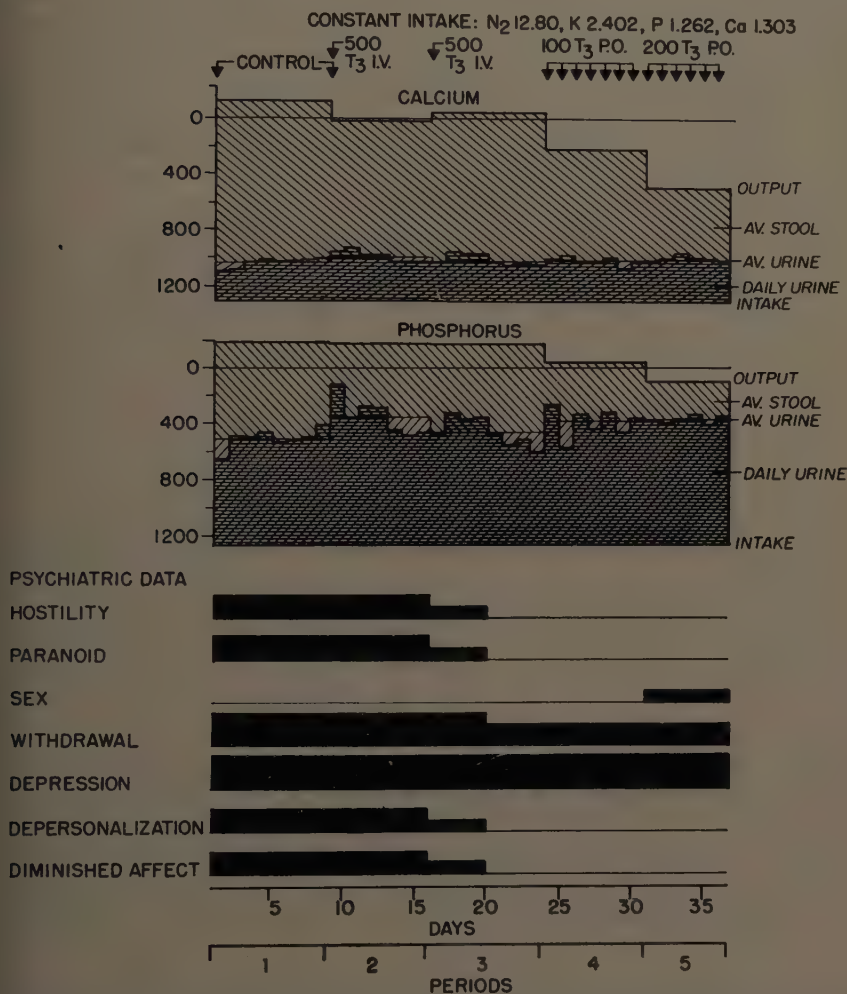


FIGURE 5. The metabolic and clinical effects of triiodothyronine in patient E, a 40-year-old female with a chronic paranoid schizophrenic reaction. Reproduced with permission from *The Journal of Clinical Endocrinology and Metabolism*.¹⁰

Urinary phosphorus excretion increased within 24 hours to 1.166 gm., remaining elevated for 3 days.

The metabolic response of this patient to triiodothyronine was characterized by a lack of any rise in urinary calcium. Following this experiment the patient was placed on triiodothyronine in daily oral doses of 200 µg. for 19 days. No significant emotional changes occurred in association with triiodothyronine administration. Balance determinations were done simultaneously, and

no significant change in calcium retention or loss resulted from triiodothyronine administration.

Patient G (PWC-10). This 34-year-old man was diagnosed as having a schizophrenic reaction of the undifferentiated type. He presented a 1-year history of progressive apathy, recurrent anxiety, withdrawal, and some depression of mood. His BMR was -9 per cent. The PBI was $4.7 \mu\text{g.}/100$

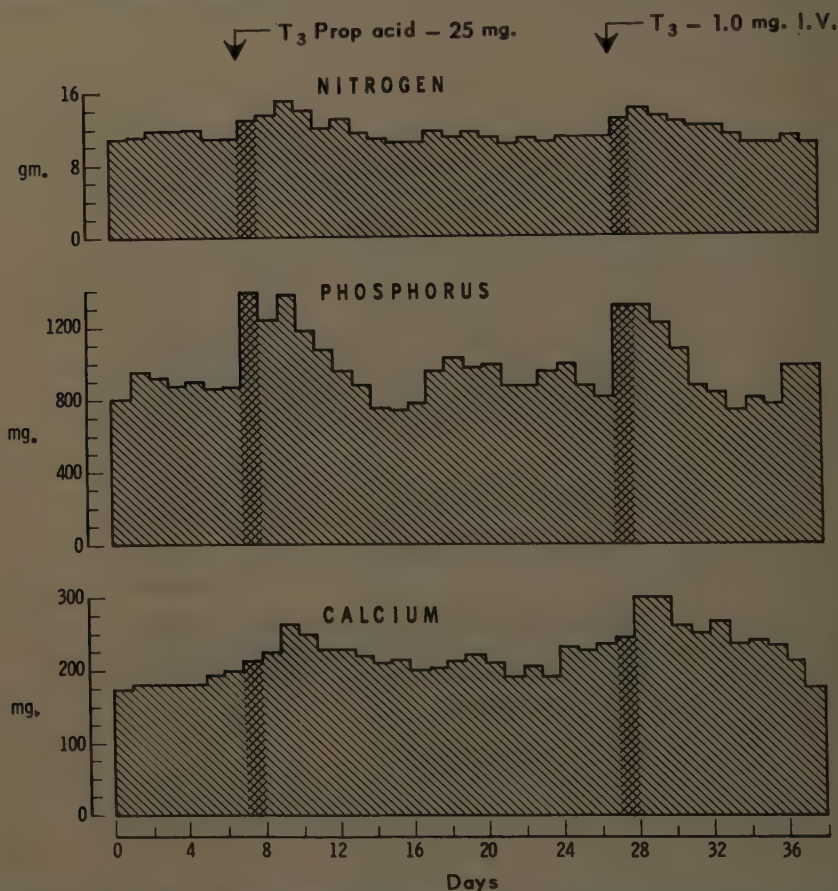


FIGURE 6. The metabolic effects of intravenous triiodothyronine and triiodothyropropionic acid in patient G, a 34-year-old male with an early undifferentiated schizophrenic reaction.

ml. He was maintained on a constant daily diet containing 12.76 gm. nitrogen, 0.801 gm. calcium, and 1.201 gm. phosphorus. During the control period daily urinary nitrogen excretion averaged 11.41 gm., urinary calcium 0.182 gm., and urinary phosphorus 0.886 gm.

On the twenty-second day of study the patient received 25 mg. of triiodothyropropionic acid intravenously; the metabolic findings are shown in FIGURE 6. Within 24 hours urinary nitrogen excretion increased to 12.70 gm., remaining elevated for 6 days. Urinary calcium increased promptly to 0.214 gm.,

reaching a peak of 0.267 gm. and remaining elevated for 9 days. Urinary phosphorus increased within 24 hours to 1.411 gm., this elevation continuing for 5 days.

Three weeks after this procedure 1000 μ g. triiodothyronine was given intravenously. Within 24 hours urinary nitrogen increased to 12.70 gm., remaining elevated for 6 days. Urinary calcium increased to 0.244 gm. within the first 24 hours, reaching a peak of 0.300 gm. and remaining elevated for 9 days. Urinary phosphorus rose immediately to 1.330 gm., remaining elevated for 4 days.

In this patient, therefore, the administration of 1000 μ g. of triiodothyronine appeared to produce a similar effect on urinary nitrogen, calcium, and phosphorus as that induced by the administration of 25 mg. triiodothyropropionic acid. In association with these procedures this patient demonstrated a limited degree of resentment, increased spontaneity and interest, and a decrease in apathy and depression of mood.

Patient H (PWC-11). This 40-year-old man was diagnosed as having a schizophrenic reaction of the paranoid type of 16 years' duration. For at least 10 years his psychopathological state had been characterized by apathy, withdrawal, incommunicativeness, and inactivity. His BMR was 0 per cent. The PBI was 4.4 μ g./100 ml. At the time of his admission to the hospital his thyroidal uptake of I^{131} was 97 per cent, with a conversion ratio of 20 per cent. When this study was repeated 6 weeks later and prior to the administration of thyroid substances the uptake had decreased to 17 per cent, with a conversion ratio of 17 per cent. He was maintained on a constant daily diet containing 12.18 gm. of nitrogen, 0.800 gm. calcium, and 1.201 gm. phosphorus. During the control period daily urinary nitrogen excretion averaged 11.13 gm., urinary calcium 0.236 gm., and urinary phosphorus 0.825 gm.

On the eleventh day of study 25 mg. triiodothyropropionic acid was given intravenously; the metabolic effects are shown in FIGURE 7. No changes in urinary nitrogen or calcium excretions were observed. During the 24-hour period following the injection urinary phosphorus rose to 1.300 gm., but this effect had disappeared by the next day. Seventeen days later a second intravenous dose of 25 mg. of triiodothyropropionic acid was administered. Again, no changes in urinary nitrogen or calcium excretion were seen. Urinary phosphorus again rose to 1.274 gm., but this effect disappeared within a day.

In this patient triiodothyropropionic acid failed to produce a change in the urinary excretion of nitrogen and calcium, in contrast to the metabolic changes seen in the previous case presented. Moreover, the effect on urinary phosphorus appeared to be markedly limited in duration. This patient also received triiodothyronine in daily oral doses of 200 μ g. for 18 days. No emotional changes occurred. Balance determinations were carried out; the metabolic responses are shown in FIGURE 8. No significant changes in calcium retention or loss were seen in association with oral triiodothyronine administration.

Patient I (PWC-12). This 24-year-old man was diagnosed as having a schizophrenic reaction, of the simple type, of 12 years' duration. He presented a psychopathological state characterized by apathy, tension, resentment, and withdrawal. A satisfactory BMR could not be obtained. His thyroidal

uptake of I^{131} was 25 per cent, with a conversion ratio of 6 per cent. He was maintained on a constant daily intake containing 10.36 gm. nitrogen, 0.801 gm. calcium, and 1.200 gm. of phosphorus. During the control period his

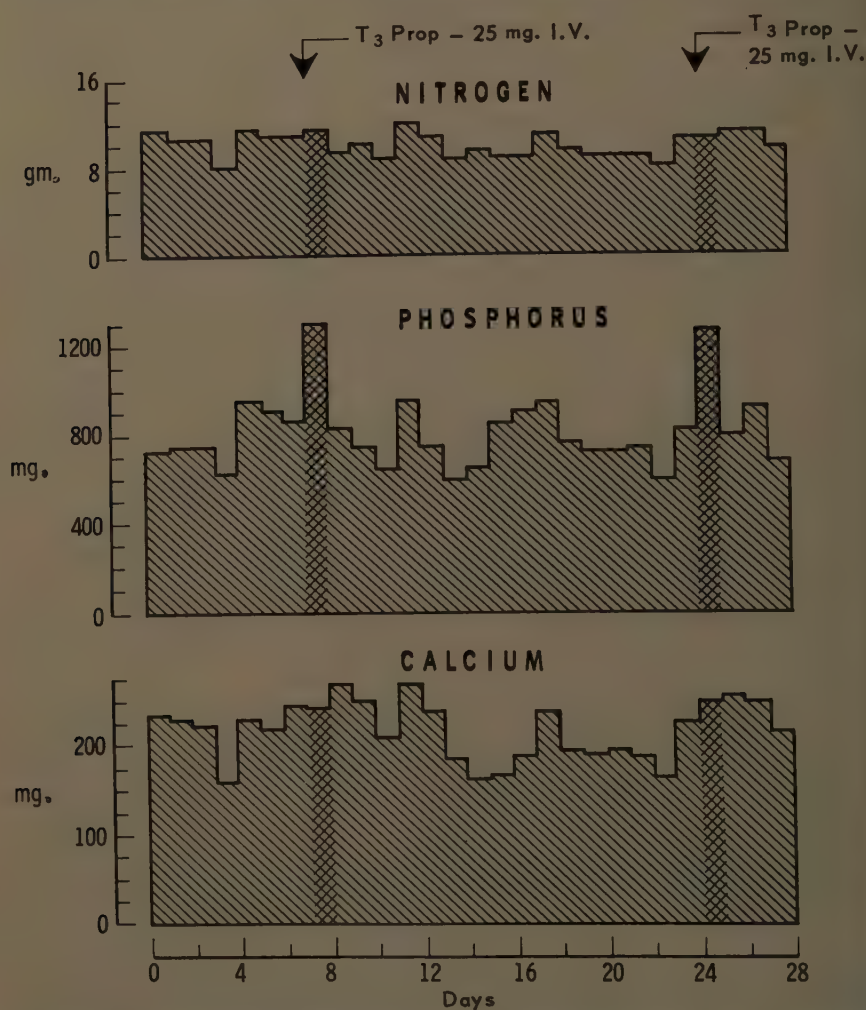


FIGURE 7. The metabolic effects of intravenous triiodothyropropionic acid in patient H, a 41-year-old male with a chronic paranoid schizophrenic reaction.

daily urinary nitrogen excretion averaged 11.0 gm., urinary calcium 0.040 gm., and urinary phosphorus 0.696 gm.

On the eleventh day of study 25 mg. triiodothyropropionic acid was administered intravenously. After a 24-hour delay urinary nitrogen increased to 12.70 gm., remaining elevated for 6 days. No change in urinary calcium was observed. Urinary phosphorus increased promptly to 1.030 gm., remaining elevated for 3 days.

Eighteen days later this patient received 1000 $\mu\text{g.}$ of triiodothyronine intravenously. After a 24-hour delay urinary nitrogen increased to 13.11 gm., remaining elevated for 5 days. After the same delay urinary calcium rose to 0.124 gm., but this effect did not persist. Urinary phosphorus increased promptly, within the first 24 hours, to 1.224 gm., remaining elevated for 3 days.

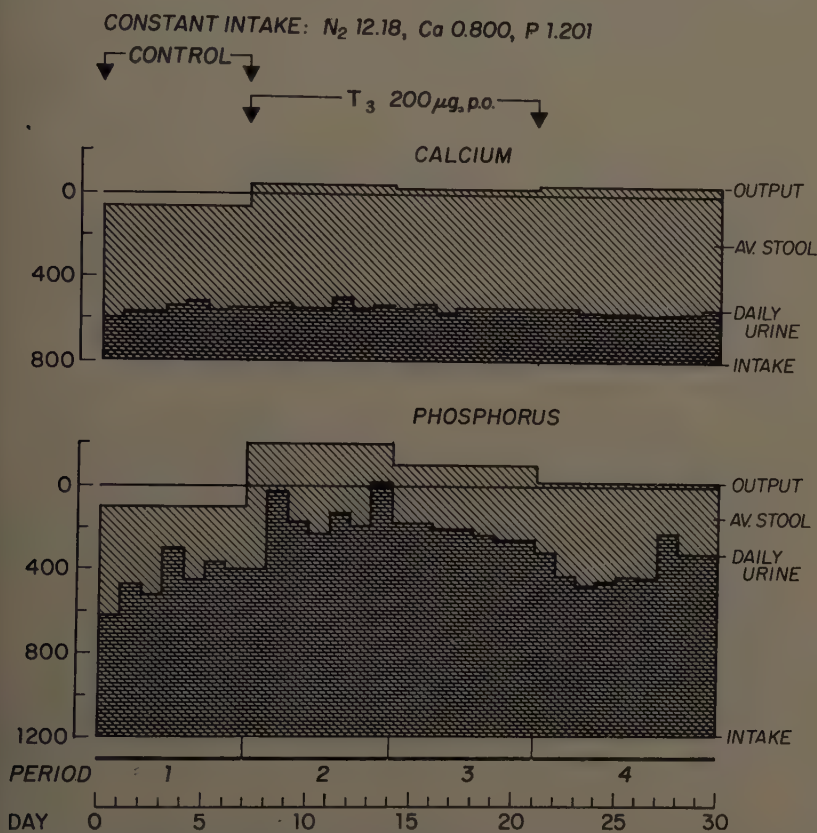


FIGURE 8. The metabolic effects of triiodothyronine in patient H, a 41-year-old male with a chronic paranoid schizophrenic reaction.

Sixteen days later the patient was again given 25 mg. triiodothyropropionic acid intravenously. After a 24-hour delay urinary nitrogen excretion increased to 12.92 gm., remaining slightly elevated for 5 days. After the same delay urinary calcium increased slightly to 0.126 gm., remaining elevated for 2 days. A slight increase in urinary phosphorus to 0.854 gm. was noted, but this effect disappeared within 1 day.

This patient consistently demonstrated a 24-hour delay before responding to the administration of either thyroid analogue with an increase in urinary nitrogen excretion. Moreover, the degree of rise in urinary nitrogen appeared to be limited. His responses to the first injection of 25 mg. of triiodothyro-

propionic acid and to 1000 $\mu\text{g.}$ of triiodothyronine seem to be almost identical. Neither analogue produced a significant increase in urinary calcium. Although an adequate rise in urinary phosphorus was noted following the first injection of triiodothyropropionic acid, this response appeared to be markedly limited when the procedure was repeated. Subsequently this patient was given triiodothyronine in daily oral doses of 100 $\mu\text{g.}$, with no evident change in his emotional state.

Patient J (PWC-14). This 52-year-old man with a diagnosis of schizophrenic reaction, paranoid type, of 20 years' duration presented a psycho-

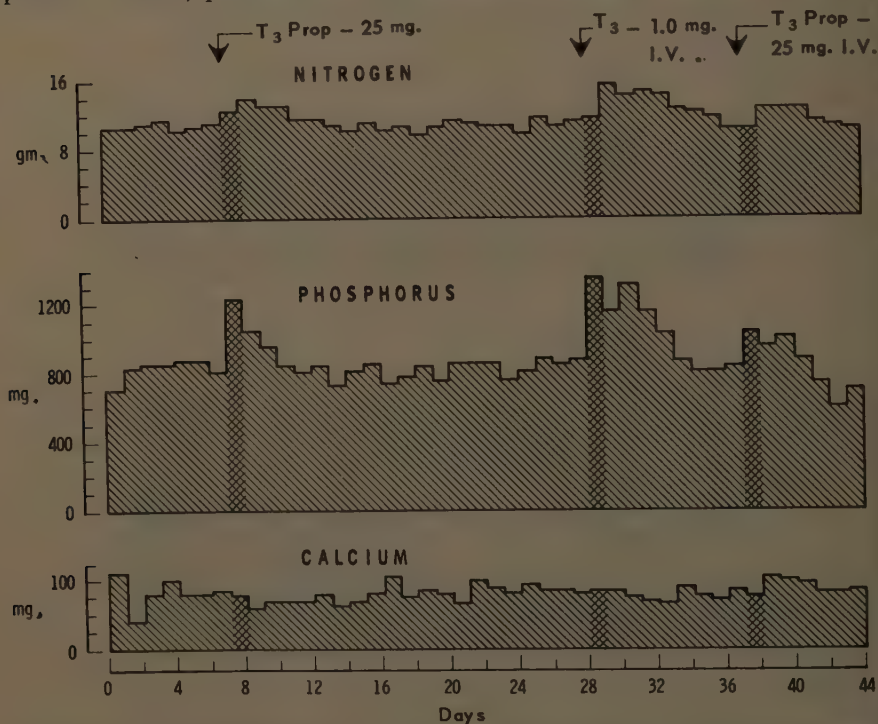


FIGURE 9. The metabolic effects of intravenous triiodothyronine and triiodothyropropionic acid in patient J, a 52-year-old male with chronic paranoid schizophrenic reaction.

pathological picture characterized by somatic delusions, depression of mood, apathy, and looseness of logical associative thought processes. His thyroidal I^{131} uptake was 29 per cent, with a conversion ratio of 27 per cent. A satisfactory BMR could not be obtained. The patient was placed on a constant diet containing 12.60 gm. nitrogen, 0.800 gm. calcium, and 1.203 gm. phosphorus. During the control period his daily urinary nitrogen excretion averaged 10.52 gm., urinary calcium 0.077 gm. and urinary phosphorus 0.818 gm.

On the thirty-eighth day of study 25 mg. triiodothyropropionic acid was administered intravenously. Urinary nitrogen rose promptly to 12.61 gm., remaining elevated for 4 days. No change in urinary calcium excretion was observed. Urinary phosphorus increased immediately to 1.230 gm., remaining elevated for 3 days. The metabolic responses are shown in FIGURE 9.

Twenty-two days after this procedure 1000 μ g. triiodothyronine was given intravenously. After a 24-hour delay urinary nitrogen increased to 15.66 gm., remaining elevated for 5 days. Again, no change in urinary calcium excretion was noted. Urinary phosphorus increased promptly to 1.349 gm., remaining elevated for 5 days.

Ten days later the patient again received 25 mg. triiodothyropropionic acid intravenously. After a 24-hour delay urinary nitrogen excretion increased to 12.68 gm., remaining elevated for 4 days. No change in urinary calcium was observed. Urinary phosphorus rose immediately to 1.028 gm., remaining elevated for 3 days.

This patient, therefore, revealed no change in urinary calcium in response to either triiodothyronine or triiodothyropropionic acid. A delay in the appearance of the nitrogen response was noted on 2 occasions, and the phosphorus response to the second dose of triiodothyropropionic acid was clearly less marked than the response to the first. No significant emotional changes were observed during the studies.

Discussion

Variations in the metabolic responses to the administration of triiodothyronine and triiodothyropropionic acid were observed among these patients. Low basal metabolic rates were observed in a number of patients studied, but the significance of these rates or of the changes in the BMR occurring during the administration of the thyroid analogues is essentially unclear. The most striking deviations from the anticipated reactions to these thyroid analogues consisted of: (1) a 24-hour delay before any increase in urinary nitrogen, calcium, or phosphorus occurred, and (2) a total absence of effect on the urinary excretion of nitrogen or calcium.

Patients A and B demonstrated a prompt increase in urinary nitrogen, calcium, and phosphorus following the administration of triiodothyronine. Patient G revealed a prompt increase in nitrogen, calcium, and phosphorus following the administration of triiodothyronine and triiodothyropropionic acid.

Four patients revealed a 24-hour delay before demonstrating a rise in urinary nitrogen following the first (patients I and J) or second (patients C and D) injection of triiodothyronine. Three patients revealed a 24-hour delay prior to the increase in urinary calcium after the second (patients C, D, and E) injection of triiodothyronine.

Two patients revealed a 24-hour delay before demonstrating a rise in urinary nitrogen following the first (patient I) or second (patient J) injection of triiodothyropropionic acid.

Patient E failed to demonstrate any increase in urinary nitrogen after the administration of triiodothyronine, and patient H failed to show any rise in urinary nitrogen following the injection of triiodothyropropionic acid. Three patients (F, I, and J) failed to demonstrate a significant increase in urinary calcium excretion following triiodothyronine administration, and 3 patients (H, I, and J) failed to show any rise in urinary calcium following the administration of triiodothyropropionic acid.

Two patients (C and D) showed less of a rise in urinary phosphorus follow-

ing the second injection of triiodothyronine than they did after the first dose. Patient E failed to demonstrate a significant rise in urinary phosphorus following the second injection of triiodothyronine. Two patients (I and J) revealed a decrease in the degree of the phosphorus response to the second injection of triiodothyropropionic acid. Patient H demonstrated an immediate but extremely brief rise in urinary phosphorus following the administration of triiodothyropropionic acid.

These observations may be considered in relation to the nature and duration of the patients' illnesses, as well as to their emotional and metabolic responses to the sustained oral administration of triiodothyronine. Those patients (A, B, and G) who demonstrated a prompt rise in urinary nitrogen, calcium, and phosphorus and those patients (C and D) whose inadequate reactions to the intravenous administration of these thyroid analogues consisted chiefly of delays prior to the appearance of urinary changes, presented symptoms of schizophrenic reactions of comparatively short duration, ranging from 4 months to 6 years. Each of these 5 patients subsequently improved clinically. Two of them (B and C) also received oral triiodothyronine daily for several weeks, leading to an increase in fecal calcium excretion and the establishment of a negative calcium balance. On this regime, each demonstrated marked emotions, in particular hostility and sexual unrest.

Those patients (E, F, H, I, and J) whose inadequate responses to the administration of these thyroid hormones were more striking, consisting in part of a total absence of urinary nitrogen or calcium changes or both, presented relatively intractable schizophrenic conditions characterized by long-standing apathy, withdrawal and, in certain cases, paranoid features. These illnesses ranged in duration from 12 to 20 years. Three of these patients (E, F, and H) also received oral triiodothyronine daily for several weeks. Two of them (F and H) failed to show any change in calcium balance or fecal calcium excretion in response to this procedure; the third (patient E) demonstrated a decrease in fecal calcium and increased calcium retention. Patients F and H revealed no emotional changes during triiodothyronine administration, while patient E showed a decrease in hostility, paranoid features, and depersonalization, and also a slight increase in sexual unrest.

These findings suggest that a defect in the metabolism of thyroid analogues may be associated with certain schizophrenic conditions, and that this effect may be more striking when the condition is intractable and has persisted for many years.

Summary

In order to explore possible relationships between thyroid function and psychopathological disorders, 2 thyroid analogues, triiodothyronine and triiodothyropropionic acid, were administered intravenously to a series of 10 psychiatric patients, 9 of whom represented various types and stages of schizophrenic states. The effect of this procedure on the urinary excretion of nitrogen, calcium, and phosphorus was determined. The metabolic effects of 1000 μ g. triiodothyronine and 25 mg. of triiodothyropropionic acid appeared to be nearly identical within individual patients. A delay of 24 hours was frequently noted prior to the appearance of urinary changes following the ad-

ministration of these analogues. Certain patients failed to respond with any increase in urinary nitrogen or calcium or both following the administration of these analogues. The possible meaning of these observations is discussed.

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THE EFFECT OF THYROID HORMONES ON ADRENAL STEROID METABOLISM

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The mechanism of action of the thyroid hormone at a molecular level is, at the present time, completely unknown.[†] Nevertheless, it is possible to gain an insight into some physiological effects of thyroxine, even though these are secondary to more basic events. A most interesting action of thyroxine is to affect the metabolism of the steroid hormones and, because they are powerful biological reagents themselves, the activity of the thyroid hormone is thereby amplified many times. The present communication is concerned with an effect, studied in rats, of thyroxine administration on the metabolism of various steroid hormones by liver enzymes.[‡]

Several reports^{5,6} have pointed out that the rate of steroid turnover in human beings is accelerated in thyrotoxicosis. Peterson⁵ has shown, in addition, that the rate of disappearance of various steroids from the plasma is accelerated in such patients. In order to understand these phenomena we must examine the enzymology involved. The α - β -unsaturated 3-ketosteroids are metabolized in the liver in 3 steps. The double bond is first saturated, and the resulting saturated 3-ketosteroid is reduced to a 3-hydroxysteroid that is finally conjugated with glucuronic acid to form a glucoside uronic acid. The latter is then excreted in the urine. The first reaction, that is, the double-bond reduction, appears to be rate-limiting, and produces an asymmetric center at carbon 5, so that 2 isomers, either 5 α or 5 β , may result. Previous work⁷ has shown that there are, in the soluble fraction of liver cells, several specific steroid reductases dependent on reduced triphosphopyridine nucleotide (TPNH) that catalyze the formation of 5 β -reduced 3-ketosteroids. More recently⁸ it has been found that a similar series of enzymes, likewise requiring TPNH, is present in liver microsomes. These enzymes catalyze the production of 5 α -reduced steroids.

Early effects of thyroxine. At a given substrate concentration either the activity of the enzyme or the amount of reduced pyridine nucleotide could control the rate of steroid reduction. TABLE 1 illustrates that liver homogenates prepared from normal rats are able to reduce the double bond of cortisone at a slow rate; this reaction is greatly stimulated by the addition of reduced TPN (added as a TPNH-generating system). Under these conditions, therefore, the level of reduced TPN can control the rate of steroid reduction. TABLE 1 also shows that unsupplemented liver homogenates from rats injected intraperitoneally with 0.5 μ mole of thyroxine per day for 3 days have a faster rate of cortisone reduction than homogenates prepared from control

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[†] A few provocative reports have appeared recently that may lead to its understanding.^{1,2}

[‡] Several discussions of this problem have appeared previously.

animals. However, when an excess of reduced pyridine nucleotide was provided, the velocity of the reaction, although greatly accelerated, was identical to that observed in the controls. This experiment illustrates that, as in the controls, the TPNH concentration could regulate the rate of steroid reduction in the thyroxine-treated animals. It shows, further, that although the total amount of enzyme involved in steroid reduction in both control and treated animals was the same, the amount of TPNH available for this process in unsupplemented homogenates was greater in the injected rats.

TABLE 1

THE EFFECT OF THYROXINE ADMINISTRATION FOR THREE DAYS ON THE REDUCTION OF CORTISONE BY RAT LIVER HOMOGENATES*

	Experiment 1		Experiment 2	
	Control	Injected	Control	Injected
	μ moles cortisone reduced/gm. liver			
No additions	7	34	10	35
With TPNH-generating system	198	207	208	215

* Homogenates were prepared from the livers of male Sprague-Dawley rats injected intraperitoneally with 0.5 μ mole of thyroxine per day for 3 days.⁴ The reaction was carried out for 5 min. at 37° C. and, where indicated, TPNH was generated with a TPN-isocitrate system as described elsewhere.⁴ The extent of steroid reduction was determined by observing the fall in O.D. at 240 $m\mu$ (see McGuire *et al.*⁴).

TABLE 2

EFFECT OF GLUCOSE-6-PHOSPHATE AND TPN ON THE RATE OF CORTISONE REDUCTION BY LIVER HOMOGENATES FROM CONTROL AND THYROXINE-INJECTED RATS

	Additions			
	None	25 μ moles G-6-P	0.5 μ mole TPN	G-6-P + TPN
	μ moles cortisone reduced/min./gm. liver			
Control	10	7	92	121
Thyroxine-injected	35	32	120	185

The reaction and assay were carried out as indicated in TABLE 1, except that the thyroxine injection was continued for 4 days and the additions were as noted above.

A possible source of this increase in available TPNH was the glucose-6-phosphate dehydrogenase reaction, the level of which, as shown by Glock and McLean,⁹ is elevated in the livers of thyroxine-injected animals. In order to demonstrate a connection between this increase in *Zwischemferment* activity and the enhanced rate of steroid reduction in thyroxine-treated animals, it must be shown that the level of glucose-6-phosphate dehydrogenase controls the availability of reduced TPN for the steroid reductase reaction. This was accomplished as follows (TABLE 2): the rate of steroid reduction was compared in liver homogenates obtained from normal and injected animals. The addi-

tion of glucose-6-phosphate and TPN to these homogenates produced a large increase in the rate of 4-5 double-bond saturation in both. However, as illustrated in TABLE 2, the homogenate from the thyroxine-treated rat was always more active than the normal. If an additional TPNH-generating system were added, the maximum velocities were the same in both preparations (TABLE 1). The level of glucose-6-phosphate dehydrogenase, therefore, could control the amount of TPNH for the reductase reaction, and we may conclude that the increase in glucose-6-phosphate dehydrogenase in injected rats could account for the augmented rate of steroid reduction in unsupplemented liver homogenates prepared from thyroxine-treated animals.

TABLE 3
THE EFFECT OF PROLONGED THYROID ADMINISTRATION ON THE
RATE OF STEROID REDUCTION

	Control	Experiment 1	Experiment 2
	μ moles cortisone reduced/gm. liver		
No additions	12	72	43
With TPNH-generating system	212	452	449

Conditions are as described in TABLE 1, except that rats were injected for 20 days.

TABLE 4
DISTRIBUTION OF STEROID REDUCTASE ACTIVITY* IN LIVER

	Control	Thyroxine-treated for 29 days
Homogenate	225	455
Particulate fraction	98	299
Soluble fraction	115	110

* After centrifugation at 100,000 g for 60 min., the particulate and supernatant fractions were assayed as described above with excess TPNH. The activity is expressed as μ moles of cortisone reduced/min./gm. of liver.⁴

Later effects of thyroxine administration. If thyroxine injections were continued at the same level, that is, 0.5 μ mole/day intraperitoneally for from 2½ to 3 weeks, an additional effect was noted that is described in TABLE 3. In this experiment the rate of cortisone reduction varied as a function of added TPNH, as in the previous experiments. In the absence of added TPNH the rate observed with liver homogenates from untreated animals was only about one third as rapid as that of a treated animal. When a TPNH-generating system was added, the rate of cortisone reduction increased in both cases; however, the situation was no longer the same as when the animals had received thyroxine for only a few days. In this case, no matter how much reduced TPN was added to the homogenates of the control animals, steroid reduction did not proceed as rapidly as in the thyroxine-treated liver homogenates. There was, therefore, in addition to the previously noted increase in glucose-6-phosphate dehydrogenase, an additional change in homogenates from animals treated with thyroxine for longer periods. The nature of this second alteration

was evident from the fact that the maximum velocity of steroid reduction, at TPNH concentrations where the reductases were saturated with reduced pyridine nucleotide, was greater in the thyroxine-treated rats. This signified that the steroid reductase enzyme levels must themselves be elevated in the livers of these animals. When they were assayed, the microsomal reductases (5α) were found, in fact, to be more active in the livers of injected rats than in those of normal ones (TABLE 4). This increase in the microsomal reductases affected not only the reduction of cortisone, but also of cortisol, 4-androstene-3,17-dione, desoxycorticosterone, and compound S. There was no corresponding increase in the soluble steroid reductases (5β). In view of the increased relative activity of the α -reductases, one might expect a concomitant increase in the amount of α -reduced product that Bradlow and his co-workers⁹ have demonstrated in the urine of patients given triiodothyronine.

Discussion

There are, therefore, at least 2 effects of thyroxine on steroid hormone metabolism: first, an increase in the rate of the TPNH-dependent double-bond reduction (both 5α and 5β) as a result of an increase in *Zwischenferment* activity and, second, an increase in the level of the microsomal 5α reductases. Since such adaptive mechanisms presumably have survival value for the organism, we might inquire, tentatively, of course, what physiological "purpose" they serve. It has been shown¹⁰ that the rate of synthesis of adrenal corticoids, and probably the other steroid hormones, is determined by the TPNH concentration in the adrenal. We have determined (unpublished data) that the adrenal glucose-6-phosphate dehydrogenase increases in response to thyroxine injection, similar to the liver enzyme, thereby providing an enhanced TPNH supply in the adrenal. One might predict, on this basis, an increase in the rate of steroid hormone biosynthesis in hyperthyroid states. Significantly, Peterson⁵ has found an increased turnover of steroid hormones, that is, an increase in the rates of both synthesis and degradation, in thyrotoxic patients. If the rate of steroid synthesis is increased, degradation should also be accelerated to prevent an excess of circulating, physiologically active hormone. This may be the reason why the TPNH-linked steroid reductase reactions are more active in thyrotoxicosis.

An increase in the rate of double-bond saturation, however, could be effected simply by providing a greater supply of TPNH, as illustrated above. Furthermore, if steroid reduction were merely a degradative reaction by which biologically active molecules were inactivated, there would be no obvious necessity for an increase in the levels of the reductases themselves. However, since the affinities of both the α and β reductases for TPNH are similar,^{7,11,12} such an elevation of reduced pyridine nucleotide would favor the production of both 5α - and 5β -reduced isomers. The recent discovery of Kappas *et al.*¹³ that 5β -reduced steroids such as etiocholanolone have the ability to induce fever in humans may provide a reason for the specific augmentation of the 5α reductases. In hyperthyroidism the affected individual is already hypermetabolic, and it would seem desirable to shunt steroid metabolism away from the pyrogenic 5β steroids toward the inactive (in this regard) 5α compounds in an attempt to lower the metabolic rate.

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THE EFFECTS OF THYROID HORMONES ON THE METABOLISM OF STEROIDS*

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This study is concerned with observations on the influence of thyroid hormones on the peripheral transformation of steroids in human subjects. The interrelation of these hormones was suggested by the fact that patients with untreated myxedema exhibited an extreme diminution in androsterone formation when compared with normal subjects. Furthermore, it was found that triiodothyronine could increase the production of androsterone in euthyroid subjects as well as in patients with myxedema.¹

The studies to be described fall into three groups: (1) the definition of the quantity and kind of endogenous androgen metabolites in myxedematous, euthyroid, and hyperthyroid subjects; (2) the effect of deficiency or excess of thyroid hormone on the metabolism of exogenous androgen; and (3) investigation of the "thyromimetic" effect of androsterone.

The steroid hormone metabolites that have been examined in this study are shown in FIGURE 1. Both of the compounds formulated, androsterone and etiocholanolone, are major end-products of androgen metabolism in man. They are structurally identical except for the orientation of the hydrogen atom attached to C-5, which is α in androsterone and β in etiocholanolone. This might seem to be a minor chemical difference; it actually represents a very significant change in the shape of the molecule. Because of this structural dissimilarity each compound has a profoundly different biological activity. Three major potential precursors of these metabolites are also shown in FIGURE 1, and it should be emphasized that these hormones are secreted both by the adrenal gland and by the testes.

In this presentation we are concerned primarily with the quantitative relationship between these two products of androgen metabolism. In order to clarify this relationship we have employed the term androsterone fraction. This value represents the percentage of the total amount of androsterone plus etiocholanolone that is comprised by androsterone alone.

The effect of hypothyroidism on androgen metabolism is shown in FIGURE 2. We have compared a group of control subjects matched, in so far as possible, as to age and sex with a group of patients with myxedema. We have studied the endogenous production of these metabolites from their precursors in each group by chromatographic methods that have been well standardized and used for many years in our laboratories.^{2,3} We have studied similarly both groups of patients with respect to their ability to metabolize a tracer dose of parenterally administered testosterone-4-C¹⁴. For this purpose the radioactive hormone was administered intravenously, and complete urine collections were

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obtained for 48 hours following the dose. After hydrolysis of the conjugates the two metabolites, androsterone and etiocholanolone, were isolated by the technique of reverse isotopic dilution.⁴ This procedure gives a very precise

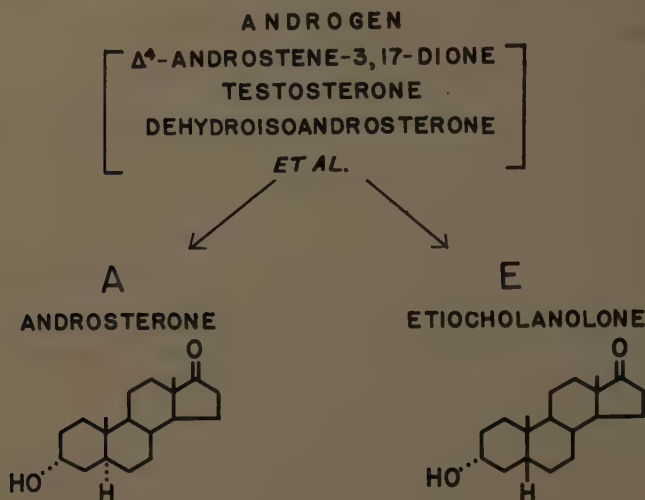


FIGURE 1. The major metabolites of androgen in man.

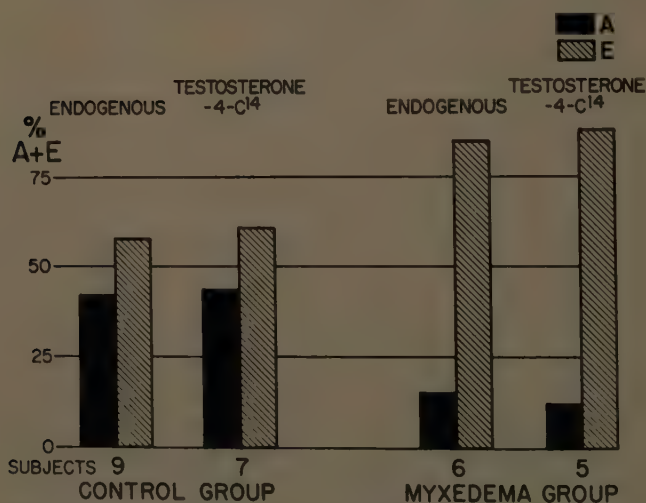


FIGURE 2. The effect of hypothyroidism on androgen metabolism.

measure of the amount of each of these compounds produced from the tracer dose of hormone. In the control group the average production of the 2 metabolites, androsterone and etiocholanolone, in 9 subjects showed an androsterone fraction of about 40 per cent. Seven of these subjects received tracer doses of testosterone-4-C¹⁴, and the results with this exogenous hormone were almost identical with the endogenous production; that is, the androsterone

fraction again was about 40 per cent, while the etiocholanolone amounted to about 60 per cent of the total of these two substances. It is clear from these results that the tracer dose of exogenously administered radioactive hormone faithfully reflected the metabolism of the hormone produced from glandular sources. Thus the metabolic sequences concerned in the production of the metabolites can be measured either from endogenously produced hormone or from labeled testosterone.

It is immediately evident from FIGURE 2 that the patients with myxedema exhibited a sharp difference in hormone metabolism from both endogenous and exogenous sources. In either case the androsterone fraction was 15 per cent or less with, of course, a concomitant increase in etiocholanolone. It is evident,

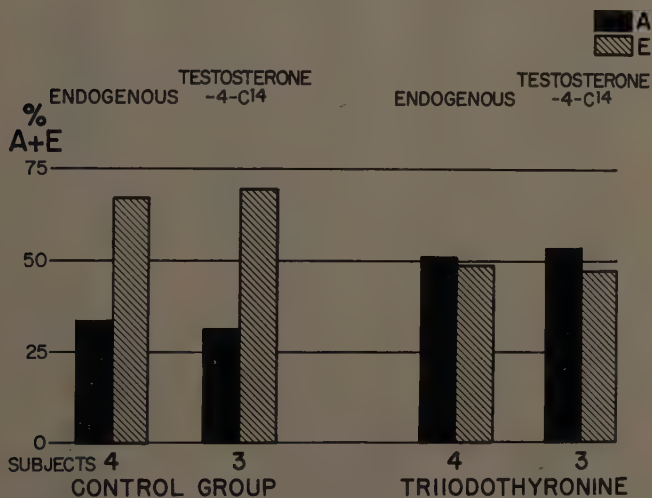


FIGURE 3. The effect of triiodothyronine on androgen metabolism.

then, that deficiency in thyroid hormone production is associated with a very considerable decrease in the production of androsterone.

FIGURE 3 demonstrates the effect of triiodothyronine administration on androgen metabolism both from endogenous glandular sources and from exogenous testosterone-4-C¹⁴. As noted above, the exogenous testosterone-4-C¹⁴ is metabolized identically with the endogenously produced hormones in that each showed an androsterone fraction of approximately 30 per cent. When these same subjects were treated with triiodothyronine there was a marked change in the metabolism of androgen from endogenous glandular secretion, as well as from intravenously administered testosterone-4-C¹⁴, since the androsterone fractions from each of these precursors rose to more than 50 per cent under the influence of the thyroid hormone.

The influence of hyperthyroidism on endogenous androgen metabolism is illustrated in FIGURE 4. Five patients were studied, and in each instance the androsterone fraction was 50 per cent or greater. Three of these subjects were elderly, and one had been castrated for cancer of the prostate. In the light

of other results,⁵ the very high androsterone fractions in these individuals is striking, but is quite in accord with the concept that has been developed in

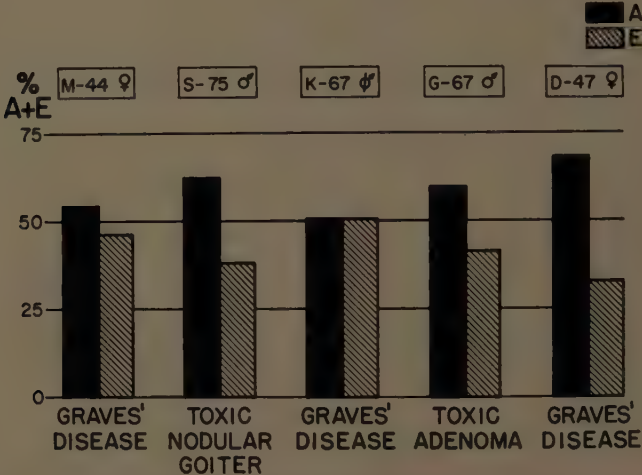


FIGURE 4. The effect of hyperthyroidism on endogenous androgen metabolism.

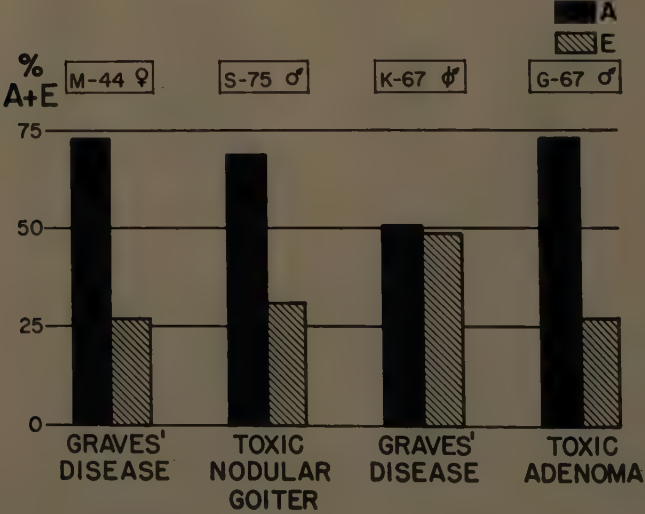


FIGURE 5. The effect of hyperthyroidism on exogenous androgen metabolism.

this study of the interrelationship of functional thyroid level and androgen metabolism.

The effect of hyperthyroidism on exogenous testosterone metabolism in four of these same patients is shown in FIGURE 5. Again it is evident that the androsterone fraction derived from testosterone-4-C¹⁴ was large in each instance. FIGURE 6 shows the effect of treatment of the hyperthyroidism on the metabolism of androgens. Two patients were studied before and after their hyper-

thyroidism had been corrected. It is evident that there was a very significant diminution in the androsterone fraction in the euthyroid state as compared with the untreated hyperthyroidism.

These results are all consistent with the view that androgen metabolism is interrelated with the level of thyroid function. These findings led us to make

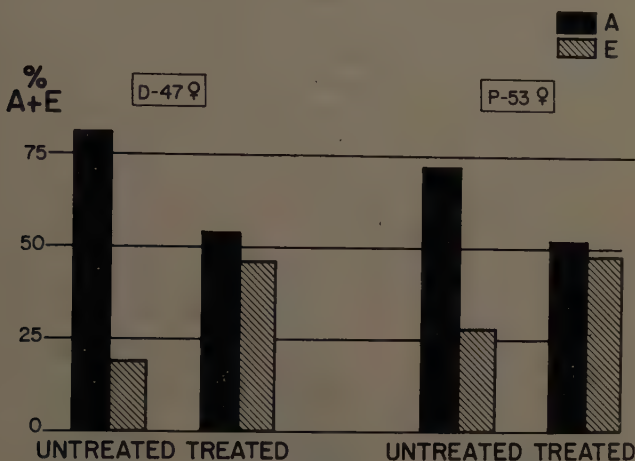


FIGURE 6. The effect of treatment of hyperthyroidism on androgen metabolism.

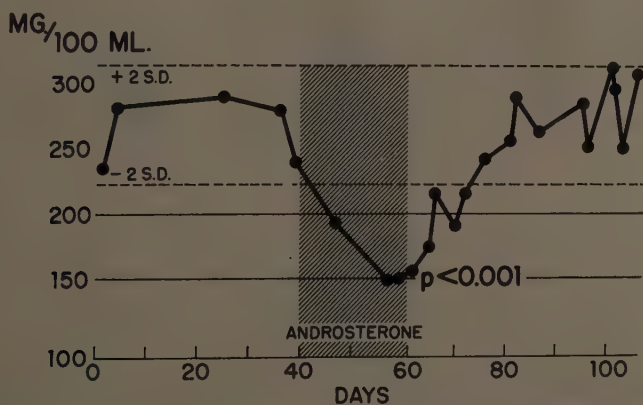


FIGURE 7. The effect of androsterone on serum cholesterol in a representative patient with myxedema.

the hypothesis that some of the recognizable effects of thyroid deficiency or excess might be mediated by the availability of androsterone in the body. One of the most evident biochemical alterations associated with thyroxine excess or deficiency is the change in the level of serum cholesterol. For this reason, we investigated the effect of androsterone on the serum cholesterol concentration in 4 patients with myxedema, in 7 patients with hypercholesterolemia, and in 5 normocholesteremic subjects. The steroid was given in a daily dose of 50 mg. intramuscularly; a representative result is shown in FIGURE 7. It is

evident that androsterone caused a sharp and significant fall in the level of serum cholesterol and that, following withdrawal of the steroid, the serum cholesterol returned to the pretreatment level. The composite data from 16 individuals treated with androsterone are charted in FIGURE 8. It is evident that there was a consistent and significant influence of this steroid hormone metabolite in lowering the serum cholesterol in the 3 classes of patients studied.

The data on which these summaries are based have been published.⁶ It is noteworthy that the androgenic hormones from which androsterone is derived have often been associated with hyperlipemic states, especially because of the increased incidence of atherosclerosis in men. Since androsterone production normally declines with age,^{2,5} and in view of the influence this metabolite exerts on the cholesterol level as shown in this study, it seems reasonable to suggest that these events may be causally associated. Obviously, androsterone is not the sole factor concerned with the level of serum cholesterol; nevertheless, a

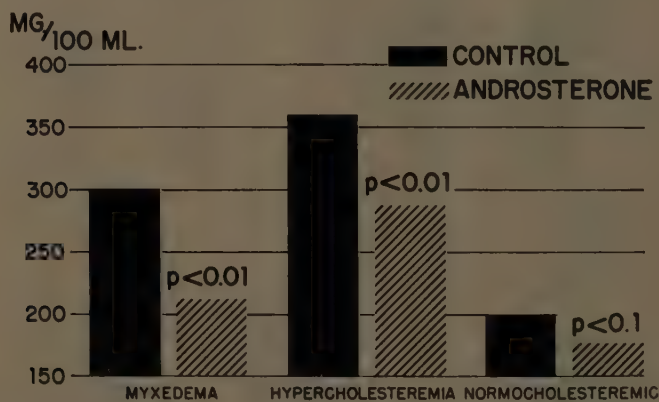


FIGURE 8. The effect of androsterone on serum cholesterol; composite data from 16 patients.

deficiency of this substance may well be one of the mechanisms involved in the pathogenesis of hypercholesteremia and, perhaps, of atherosclerosis.

The most important conclusion that emerges from these studies is that metabolites of hormones may have an independent physiological function that is in no necessary way related to that of the glandular secretory products from which they are derived. We have recently demonstrated that etiocholanolone, the other major androgen metabolite, is a pyrogen in man.⁷ This hitherto unsuspected property may have physiological significance, as suggested from the findings of Bondy and his associates,⁸ who have indicated that a disturbance of etiocholanolone metabolism could be associated with the presence of fever in periodic disease. It is thus strongly suggested that metabolites are not simply inactive end products of a spent hormone, but may have their own important functions. There is a significant corollary to this conclusion. The present studies done *in vivo* and with human subjects have not as yet been reproduced in experimental animals. Thus androsterone does not lower the hypercholesterolemia of cholesterol-fed rabbits, and Kappas (personal communication) has shown that steroids that are pyrogens in man do not produce

fever in other species. Since the metabolism of steroid hormones in man is appreciably different from that of the common laboratory animals, it is evident that studies in other species may not disclose the biological activity characteristic of the hormonal metabolites produced by the human.

Summary

Thyroid hormone level altered steroid hormone metabolism.

Some effects of the thyroid may be mediated through steroids.

Androsterone, a steroid metabolite, lowered elevated serum cholesterol.

Steroid metabolites have biological action separate from hormones and may be specifically related to disease.

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Part V. Chemistry and Physiology of the Thyroid-Stimulating Hormone

PURIFICATION AND CHEMISTRY OF THE THYROID-STIMULATING HORMONE*

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This paper describes progress made in the purification of the thyroid-stimulating hormone by the application of some of the newer techniques of protein chemistry. In our laboratory the goal has been not only to obtain material for chemical studies but to enable a correct assessment to be made of the question of whether the many effects on the thyroid that are manifested by pituitary extracts are all biological properties of one hormone or are the result of the existence of several completely different hormones (TABLE 1). The earlier work on the purification and chemistry^{1,2} was hampered by the lack of a standard for comparing results from different laboratories, a situation now remedied by the introduction of the U.S.P. thyrotropin reference substance.† In terms of this substance one can calculate (by comparison of the activity of the final product with that of the initial extract) that one of the well-described earlier preparations, that of Ciereszko,^{11,12} had a potency of about 5 U.S.P. U./mg. Ciereszko's material and material of 2 to 4 U.S.P. U./mg. prepared by Fels *et al.*¹³ had 2 common properties, that is, they were both soluble in dilute trichloroacetic acid (TCA), and both yielded ultracentrifugal data indicating relative homogeneity and a molecular weight in the range of 10,000. In contrast, recent cation-exchange chromatography, both in our laboratory¹⁴ and as reported by Condliffe and Bates,¹⁵ led to preparations which, although assaying 5 to 7 U.S.P. U./mg., had ultracentrifugal properties indicating a greater molecular weight, namely, about 30,000. This value also appears to be the correct one for material of higher potency (20 to 60 U.S.P. U./mg.) whose preparation and properties are described below. Experimental details of much of the work have been published recently.^{16,17} The principal method of bioassay has been the measurement of the uptake of P³² into chick thyroid following intracardial injection of hormone.¹⁸

In obtaining higher potency and greater purity two techniques have been particularly valuable. One is the use of anion-exchange chromatography on diethylaminoethyl-cellulose (DEAE-cellulose),¹⁹ which was introduced for studies with thyrotropin by Condliffe and Bates,²⁰ who showed that 5-U./mg. material could be fractionated further. The second is the use of electrophoresis in starch gels²¹ to investigate the chromatographic fractions.^{22,23} The procedure used in our laboratory is briefly as follows. An extraction based on countercurrent distribution studies²⁴ allows the preparation by batch procedures of 5-U./mg. material from 0.8-U./mg. concentrates prepared by Cie-

* The work reported in this paper was supported in part by grant No. C-2290 from the National Cancer Institute, Public Health Service, Bethesda, Md., and in part by the Cancer Research Funds of the University of California.

† Available from U.S.P. Reference Standards, New York, N. Y.

reszko's method.^{11,22} During the extraction a biologically active contaminant (a substance causing a transient inhibition of the milk-clotting activity of pepsin) of many thyrotropin preparations is removed.^{24,25} Two passages of the 5-U. material through columns of DEAE-cellulose gave fractions with potencies of 30 to 40 U.S.P. U./mg.²² However, the active material did not emerge as a sharp peak from the columns, and starch-gel electrophoresis at pH 9.5 showed at least 4 components to be present, all of which proved to be biologically active, when areas of the gels containing the components were cut out, extracted, and the extracts assayed.

Interpretations of Chromatographic Results with Starch-Gel Electrophoresis

FIGURE 1a shows the initial chromatography of beef thyrotropin concentrates on DEAE-cellulose. Following emergence of protein represented by the first major peak (gradient to 0.05 *M* glycine, pH 9.5), a second gradient elution (to 0.15 *M* glycine) was performed, and a second peak resulted. Bio-

TABLE 1

INVESTIGATIONS OF SOME EFFECTS OF ANTERIOR PITUITARY EXTRACTS DEMONSTRATING THE EXISTENCE OF THYROTROPIC HORMONE

Study	Investigators	Date
1. Restoration of thyroid activity in hypophysectomized amphibia and mammals	Allen ³	1916-1927
2. Histological changes	Smith ⁴	
3. Increase in thyroid weight	Loeb and Basset ⁵	1929
4. I ¹³¹ uptake in thyroids of mammals and birds	Rowlands and Parks ⁶	1934
5. I ¹³¹ release in chicks	Ghosh <i>et al.</i> ⁷	1951
6. P ³² uptake in chicks	Gilliland and Fraser ⁸	1953
7. Weight changes in thyroid slices due to fluid uptake	Borell and Holmgren ⁹	1949
	Bakke <i>et al.</i> ¹⁰	1957

logical activity was found in the second peak (Fraction IV; 20 U./mg.) and also in Fraction III (5 U./mg.). The starch-gel electrophoresis patterns (FIGURE 2a) of the fractions showed that all consisted of several components, with particularly complex patterns given by the active fractions. Similar results have been observed with sheep concentrates and are shown in FIGURES 1b and 2b.* The above results indicated that an even more gradual increase in the strength of the eluting buffer might effect improved chromatographic separation. Accordingly, rechromatography was carried out as illustrated in FIGURE 1c. After each gradient was applied, the concentration of the eluting buffer was kept constant until the amount of protein in the effluent approached zero. Several distinct peaks were obtained, although large volumes of eluting buffer were required. Starch-gel electrophoresis showed that 3 components, designated as *h*, *i*, *j*, emerged from the column first, followed in orderly progression by the components designated *a* to *f*. Bioassay of the fractions showed

* It is also of interest that studies recently published by the group at the National Institutes of Health²⁶ have shown that the material emerging without significant holdup on the columns (with 0.005 *M* glycine) contains most of the luteinizing hormone (LH or ICSH) activity found in the starting material for the chromatography.

biological activity ranging from 10 U.S.P. U./mg. to 30 to 40 U.S.P. U./mg. in Fractions III to VIII; the other fractions had only a low order of activity (1 to 5 U./mg.). Fractions III (component *b*), IV (component *c*), and V (component *c*) migrated essentially as single bands in the electrophoresis. A total of 19 mg. of *b* and 21 mg. of *c* was obtained. The yield of units represented in these 2 components as compared to the total units present in the anterior pituitaries used for this preparation is about 6 per cent, and about an equal number of units was recovered in the other active fractions. In terms of specific activity an approximately twenty-two hundredfold purification of the material from glands has been achieved (dry-weight basis). A specific activity of about 60 U.S.P. U./mg. was initially found in a fraction (containing only component *c*) from a

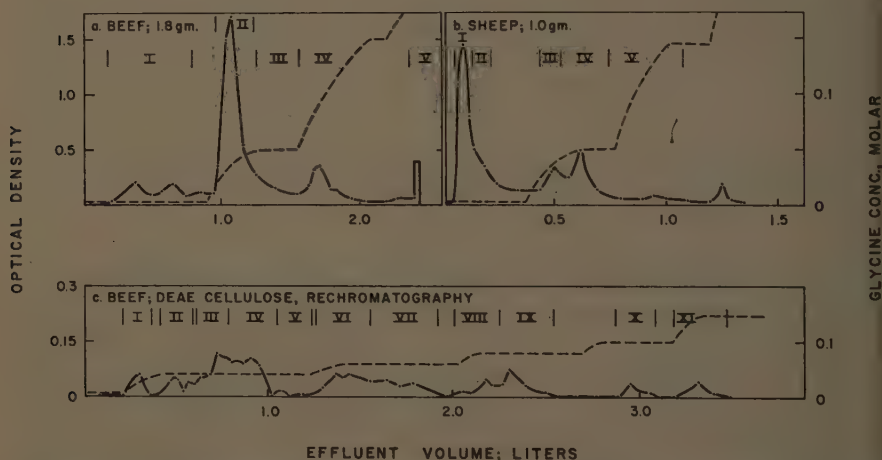


FIGURE 1. Chromatography of thyrotropin on DEAE cellulose: (a) 1.8 gm. of beef material (5 U.S.P. U./mg.) on a column 3.4×20 cm. (Fractions III and IV contain the biological activity); (b) 1.0 gm. of sheep material (5 U.S.P. U./mg.) on a column 1.9×24 cm. (Fractions IV and V contain the biological activity); (c) rechromatography of 150 mg. of material (20 U.S.P. U./mg.) corresponding to Fraction IV of (a) on a column 1.9×23 cm. The pH was 9.5; ———— represents optical density and - - - - the glycine concentration of the eluting buffers. Note that large volumes of buffers are required to complete the elution.

similar experiment. Upon storage, the more active material appears to lose potency until a level of about 20 to 30 U./mg. is reached.

Confirmation of the presence of the biological activity in each of components *a* to *f* was obtained in a number of experiments in which areas of the gels were assayed following electrophoresis. In these experiments, after vertical cuts were made in the gel edges,¹⁶ one third of the gel was cut off the top and stained for a guide. Areas of gel corresponding to the location of the stained bands were then excised, extracted, and the extracts assayed. Activity was always found in the areas occupied by components *a* to *f*, while *h*, *i*, and *j* were inactive. FIGURE 3 shows an electrophoresis and bioassay experiment with 0.2 mg. of Fraction IV, FIGURE 2c (*c*, plus traces of *b* and *d*). Approximately 90 per cent of the recovered activity was from the area corresponding to *c* and, as expected from the small amounts of the other 2 components present, 5 to 10 per cent from the areas corresponding to *b* and *d*.

Results of Experiments Designed Toward Further Fractionation

The bioassays of the various chromatographic fractions, as well as the assays of areas of starch gels containing components *a* to *f*, indicate that the thyrotropic activity is an intrinsic property of each of these components. However, such evidence is based only on the methods of fractionation and criteria of

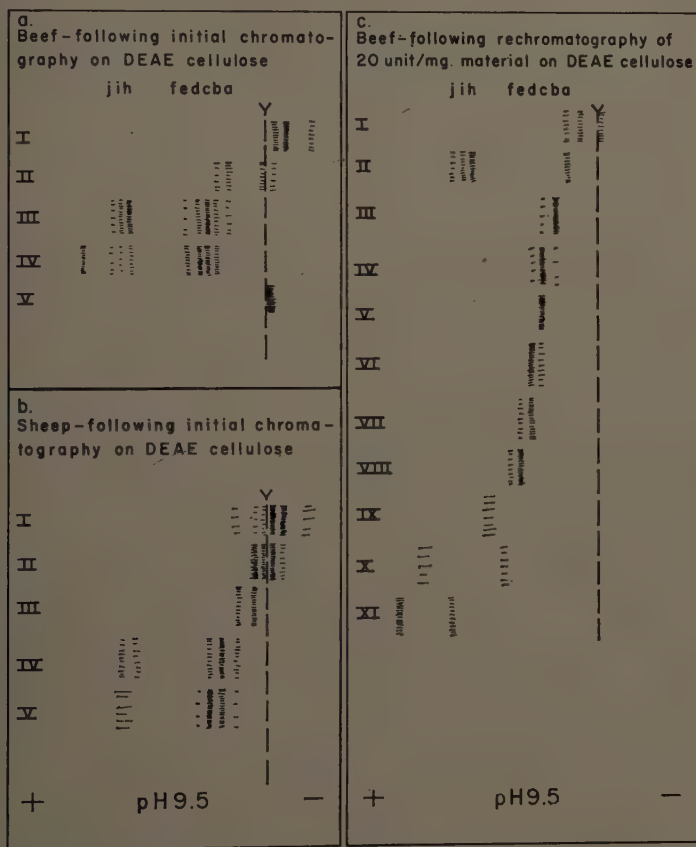


FIGURE 2. Tracings of starch-gel electrophoresis patterns of the fractions obtained after the chromatography shown in FIGURE 1a, b, and c, respectively (0.1 mg. samples of each fraction were used). The number of lines in each band indicates the relative intensity of staining.

homogeneity employed. Accordingly, a number of other experiments have been carried out designed toward further fractionation. These have included additional chromatography on DEAE-cellulose and starch-gel electrophoresis under various conditions. No separation of the activity from components *a* to *f* was achieved. To determine if the same bands could still be observed in the gels following chromatography on other media, preparations that had been once chromatographed on DEAE-cellulose were rerun on the cation exchanger, carboxymethyl-cellulose,^{15,19,26} or on hydroxyl apatite.^{27,28} In each case the chromatograms showed a greatly improved separation of *h*, *i*, and *j* from what

still proved to be the active components, *a* to *f*, and, interestingly, the active components were found to emerge from the columns not only again in stepwise progression but in the reverse order from that found with the anion exchanger.

The fact that a reverse order of emergence was observed, together with the

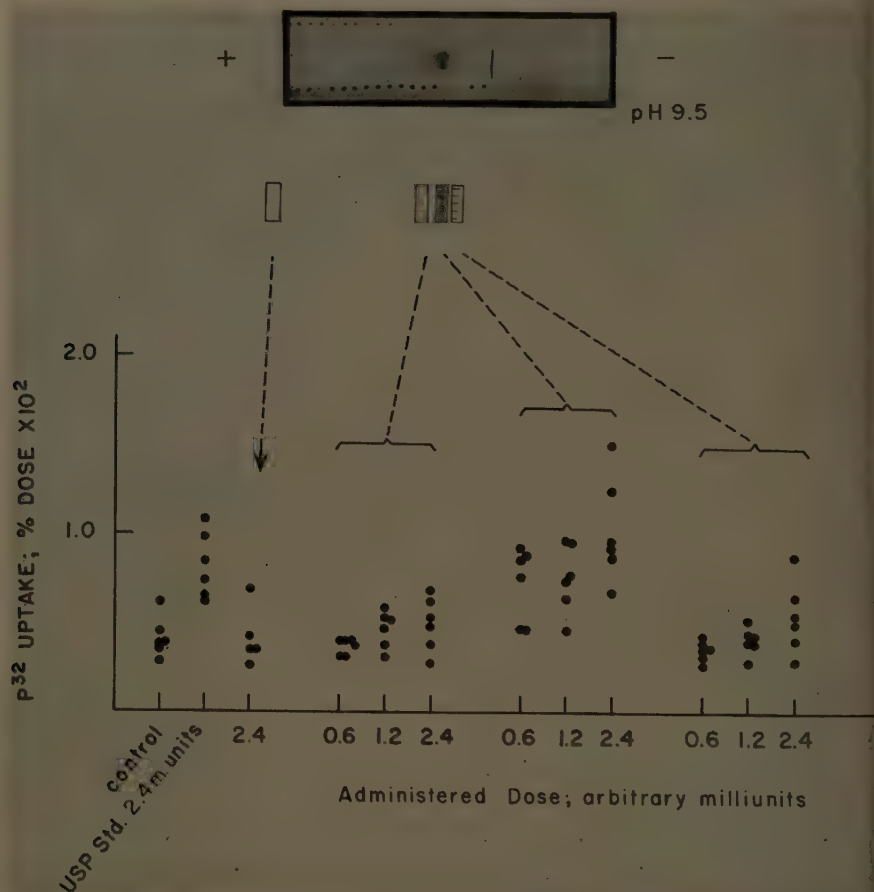


FIGURE 3. Photograph of the starch-gel electrophoresis pattern of Fraction IV, FIGURE 1c (component *c* plus traces of *b* and *d*) together with the results of bioassay performed on extracts of areas of the gel. The tracings below the photograph show the areas assayed. The dosage of each extract was determined on the previously determined basis that 50 per cent of the applied activity is recoverable from gels and on the assumption that each area tested contained all this activity. Each circle represents a single chick.

appearance of the separate bands under many different conditions, makes it highly unlikely that the bands represent artifacts of the electrophoresis. The data, however, cannot exclude the possibility that the several active components arose from a single substance during the purification procedures prior to chromatography, that is, that they represent a series of partial degradation products, all with the same biological activities. Other possibilities are that

the bands represent a series of proteins, each biologically active and each synthesized in the glands of all individuals of the species or, alternatively, that they are a series (each closely related but distinctly different) that results from genetic differences among individuals of the species. Also, the bands could represent a series of different proteins with either a small peptide or a protein of much higher specific activity bound to each by noncovalent forces. Of these possibilities, only the last is readily studied with the material and techniques presently available.

Electrodialysis Experiments with the Dialysis Membranes Implanted in Starch Gels

Electrodialysis has been used successfully with corticotropin and the posterior pituitary hormones to demonstrate that their respective biological activities do not reside in relatively large proteins but in small peptides. It

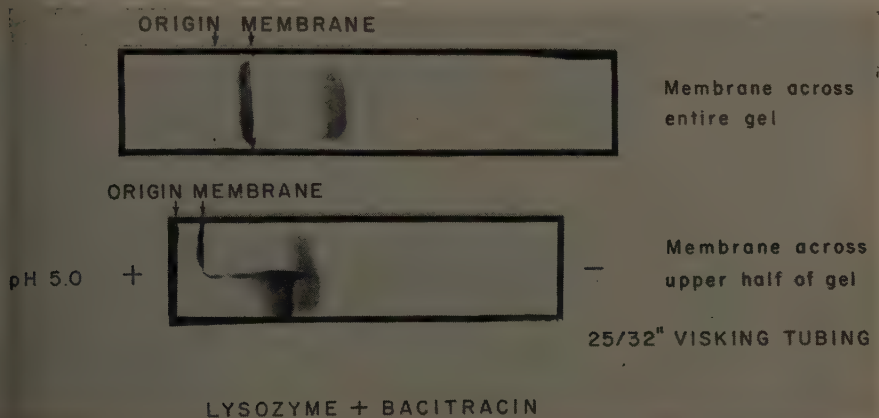


FIGURE 4. Starch-gel electrodialysis patterns showing the separation of bacitracin and lysozyme.

occurred to us that the implantation of a membrane into a starch gel might make an excellent microelectrodialysis cell for determining the passage or non-passage of thyrotropic activity through membranes of known porosity.²³ The porosity of various membranes was determined with proteins of known molecular weight, and the most suitable sizes were found in a series of Visking casings that also have been used by Craig *et al.*²⁹ in studies of the diffusion dialysis of proteins. The principle of the method is shown in FIGURE 4, which shows the separation of bacitracin, molecular weight 1400, and lysozyme, molecular weight 14,000. A $2\frac{5}{32}$ -inch Visking tubing was used; however, $2\frac{9}{32}$ -inch tubing was found most suitable for studies with thyrotropin, as with this membrane neither the biological activity nor any stainable material would pass through at pH 5.0. If, however, the membrane was stretched by hydrostatic pressure,²⁹ both activity and staining material passed through it. FIGURE 5 illustrates a typical experiment, including assay data, in which the membrane was inserted completely across the gel to prevent leakage of material around the membrane. Upon completion of a run (3 to 4 hours) the top and bottom

of the gel were carefully sliced off and stained to serve as a guide for cutting out particular areas of the gel for bioassay. A false assay due to leakage of activity along the top and the bottom of the starch-gel block was also excluded. On the left, the results with the nonstretched $20/32$ -inch tubing show that no staining material passed through the membrane; considerable activity was recovered from the area between the origin and the membrane. None of the

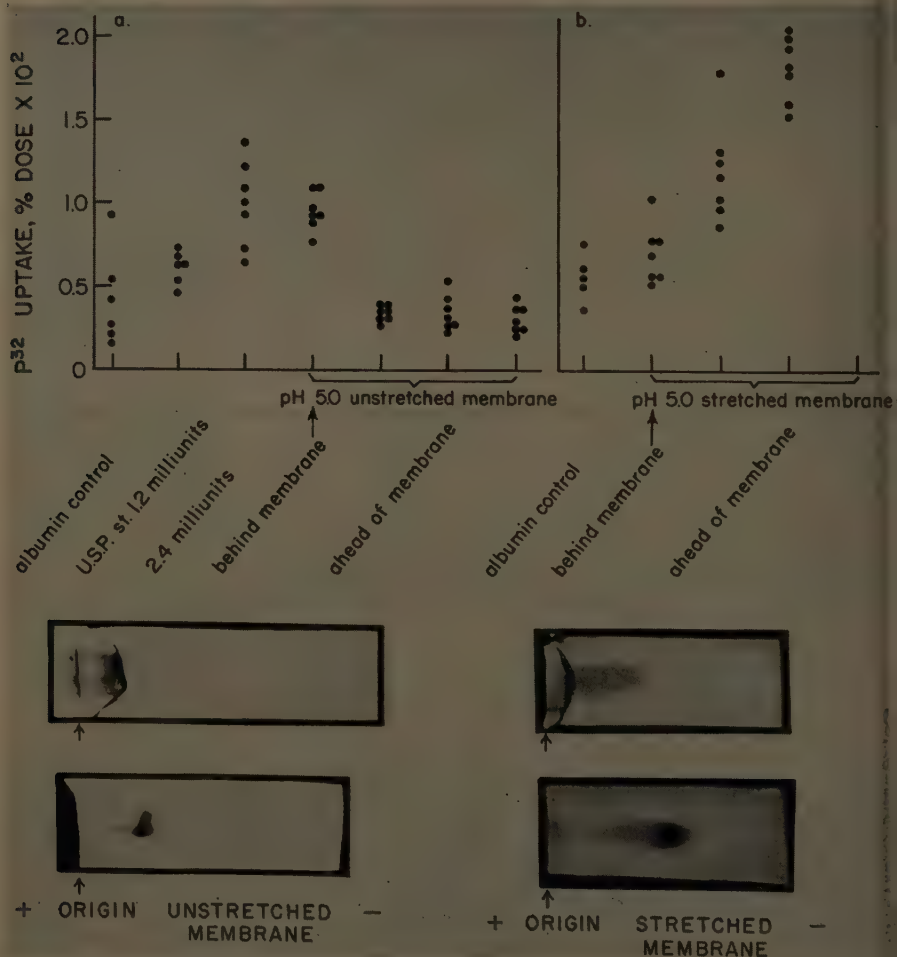


FIGURE 5. Starch-gel electrodialysis patterns of beef thyrotropins (*c* and *d*), together with bioassays showing the passage and nonpassage of thyrotropic activity through stretched and unstretched $20/32$ -inch Visking membranes. The origin of insertion of the samples (0.75 mg.) is shown, and "behind membrane" refers to the area of gel between the origin and the membrane. "Ahead of membrane" refers to areas between the membrane and the negative electrode. The lower photographs show the mobility of the samples under identical conditions but without membranes. In (*a*) 3 areas were taken ahead of membrane, and a fivefold greater dosage was given than from behind membrane. In (*b*) both an equal dose and a fivefold increase were given from the area ahead of membrane as compared to behind membrane.

3 areas beyond the membrane yielded detectable activity at 10 times the dosage. On the right it may be seen that, after stretching, the membrane allowed both stainable material and biological activity to pass. By calibration of both stretched and unstretched membranes with proteins of known molecular weight, the estimated molecular weight of the thyrotropin preparation is in the range of 26,000 to 30,000; thus, no small active peptide can be detected by this technique. Similar experiments were carried out with a series of preparations containing respectively *a*, *b* and *c*, and *e* and *f*. Identical results were obtained with respect to the passage or nonpassage of staining material. It thus appears that all the active components are in the same range of molecular size.

Because of the earlier values of 10,000 reported for the molecular weight of preparations soluble in TCA, the question of whether TCA treatment would dissociate a low-molecular-weight substance with high specific activity from the bulk of the protein was reinvestigated. When a sample containing *c* and *d* was dissolved in water and TCA and NaCl added,¹³ 2 fractions were obtained, 1 soluble and 1 insoluble. Both fractions, however, had the same biological activity and showed the same starch-gel patterns as the starting material. The TCA-soluble fraction also showed identical behavior as compared to the starting material in an electrodialysis experiment with a $20\frac{1}{32}$ -inch membrane. It is probable that the less active material reported by earlier workers consisted of a small amount of hormone plus proteins of about 10,000 molecular weight, which were soluble in TCA. The possibility remains, however, that the activity still is a property of a small molecule of high potency that has not been separated from components *a* to *f* by the techniques employed. As long as no separation is achieved, we have proposed to refer to the components as beef thyrotropins *a*, *b*, *c*, *d*, *e*, and *f*, *a* having the least electrophoretic mobility toward the positive electrode at pH 9.5.¹⁶

Comparison of Beef Thyrotropins with Preparations from Other Species

As shown in FIGURES 1*b* and 2*b*, sheep concentrates exhibit the same behavior on DEAE-cellulose columns as do beef thyrotropins; biological activity was shown to be in the same fractions, and similar starch patterns are seen. This has also been found to be true for a whale thyrotropin preparation (6 mg. of material assaying about 15 to 20 U.S.P. U./mg. were prepared from 900 gm. of whale pituitaries). Although the work on the sheep material has not been carried as far as that with beef, several active components have again been observed. They also appear to emerge from cation-exchange columns in the reverse order as from anion-exchange columns. Material from all 3 species appears to be of about the same molecular weight as shown by the electrodialysis technique (FIGURE 6). Prolactin was used as a control, and bioassay of areas behind and in front of the membranes showed that biological activity occurred only where stainable protein was observed. Ellis has also reported on the chromatography of sheep thyrotropin.³⁰

Biological Properties and Chemical Composition of Beef Thyrotropins

Although chemical synthesis is the only final proof of the isolation and chemical nature of a biologically active substance, the results of the chromatography,

electrophoresis, and electro dialysis afford strong evidence that, with respect to beef, components *a* to *f* are thyrotropins that differ only slightly from each other. Although a complete spectrum of bioassays has not been determined for each individual component (of which only *b* and *c* have been obtained by chromatography as essentially homogenous preparations), all assays thus far have agreed with the results of the measurement of P^{32} uptake into chick thyroids. The experiments are summarized in TABLE 2. It should be noted also that, when the various assays for thyrotropic activity have been carried out on other chromatographic fractions that do not overlap with the thyrotropins, little or no biological response is obtained.

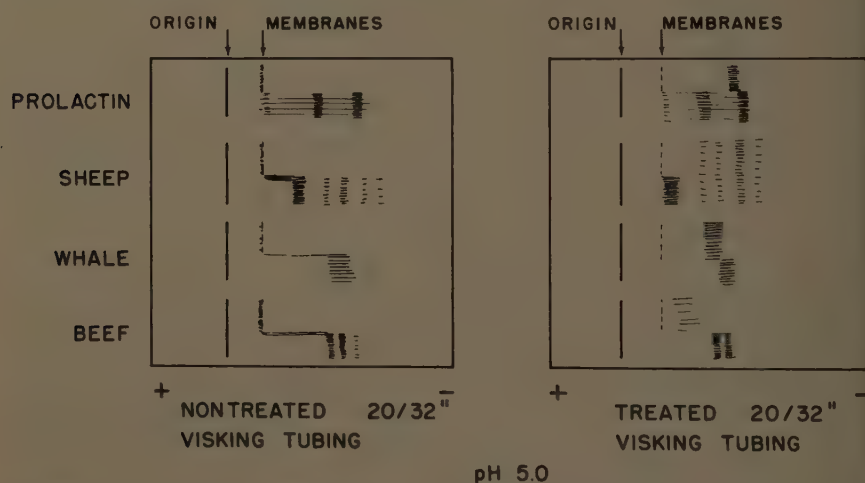


FIGURE 6. Tracings of starch-gel electro dialysis patterns showing a comparison of the passage and nonpassage of the staining components of beef, sheep, and whale thyrotropin preparations. The sheep and whale preparations had been chromatographed once on DEAE-cellulose and still contained some inactive components. Biological activity was in the bands passing through the treated membrane. The treated membranes in these experiments were made more permeable by exposure to a zinc chloride solution. The prolactin sample was a gift of the Endocrinology Study Section, National Institutes of Health, Bethesda, Md.

The first step in determining the chemical differences that must exist among the components is to determine composition. The results of amino acid analyses by the method of Moore and Stein³⁴ with 150-cm. columns of the ion-exchange resin Dowex 50 are shown in TABLE 3. Triplicate analyses of hydrolyzates of *b* and *c* were performed and show a close similarity in composition between the 2 thyrotropins. In proteins of the molecular size of the thyrotropins, however, the detection of a difference in 1 or 2 amino acid residues per molecule is difficult. Of the apparent differences observed, the most probable real difference between *b* and *c* is in the leucine content. The variation in glycine was shown to be due to residual glycine from the buffers used in the DEAE-cellulose chromatography, and the variation in cystine may be due to different degrees of destruction during hydrolysis. The high cystine content and the absence of tryptophan are of particular interest.

With respect to carbohydrate, thyrotropin activity has long been thought to be a property of a glycoprotein.* In the present work 2 hexosamines (identified as glucosamine and galactosamine by their chromatographic behavior) are found in both *b* and *c* (TABLE 3). A further study of carbohydrate has been made on a preparation purified first by DEAE-cellulose chromatography followed by chromatography on hydroxyl apatite. The preparation consisted primarily of *c* and *d*, with traces of the other active components. Milder

TABLE 2
BIOASSAYS OF BEEF THYROTROPINS

History of the preparation	Components present	Specific activity P ³² uptake (approx. U.S.P. U./mg.)	Biological activity by other assays
Chromatographed twice on DEAE-cellulose	<i>d, e</i>	40, 30	50 U.S.P. U./mg.—uptake of water by beef thyroid slices. ¹⁰ Expected stimulation of the uptake of I ¹³¹ by chick thyroids.
	<i>c, d</i>	30	40 U.S.P. U./mg.—uptake of water by beef thyroid slices.
	<i>c</i>	60; 30; 30*	Approx. 20 U.S.P. U./mg. by measurements of increase in blood iodine. ³¹
	<i>c</i>	30; 35; 40; 20*	100 µg. dose caused threefold increase in weight of chick thyroids; ³² 10 µg. (0.2 to 0.3 U.S.P. U.) caused the same weight increase as 0.2 U. of U.S.P. standard. Expected histological changes observed.
	<i>b</i>	35; 30–25*	Same changes in weight and histology of chick thyroids observed as with <i>c</i> .
Chromatographed once on DEAE-cellulose	<i>a-f</i> plus some <i>h, i, j</i>	20	Approx. 20 U.S.P. U./mg. by measurement of I ¹³¹ uptake in hypophysectomized rats.
	predominantly <i>c, d</i>	15–25	23 U.S.P. U./mg. by measurement of I ¹³¹ release from chicks. ³³
Chromatographed twice on DEAE-cellulose	<i>h, i, j</i>	<1	Inactive by measurement of increase in blood iodine.

* Each value is a separate assay. It may be seen that when the assays were repeated, the samples showed a decrease in potency to about 20 to 30 U.S.P. U./mg.

conditions of hydrolysis, which are more favorable to the preservation of carbohydrate, were used than when the amino acid analysis was performed. Glucosamine and galactosamine were again found, together with the hexose, mannose, and the methylpentose, fucose. Sialic acid could not be detected. The amounts are shown in TABLE 3, calculated as residues per 28,000 molecular weight. The total recovery in terms of amino acid, hexose, and hexosamine

* Recently Geschwind and Li have shown that treatment of crude thyrotropin preparations with periodate causes loss of most of the biological activity, due presumably to the oxidation of the carbohydrate moiety.³⁵

residues was about 85 per cent. The low recovery may result from the presence of as yet undetected constituents or from destruction during hydrolysis, a likely possibility considering the glycoprotein nature of the material.

In summary, one can say that the combination of the techniques of starch-gel electrophoresis and chromatography on various media has revealed a complex chemical situation with respect to beef thyrotropin, in that 6 closely related components possess thyrotropic activity. Whatever the chemical differences among these components may be, the evidence to date indicates that all of them possess the same spectrum of biological activities. It should be noted that similar situations have been observed with other proteins, one outstanding example being the insulin and desamidoinsulin discovered by Harfenist and

TABLE 3
AMINO ACID AND CARBOHYDRATE COMPOSITION OF BEEF THYROTROPINS
(Calculated for a Molecular Weight of 28,000)

Constituent	Thyrotropin <i>b</i> No. of residues	Thyrotropin <i>c</i> No. of residues	Constituent	Thyrotropin <i>b</i> No. of residues	Thyrotropin <i>c</i> No. of residues
Aspartic acid	14	14	Leucine	8	7
Threonine	15-16	15-16	Tyrosine	10-11	10-11
Serine	10	10Y	Phenylalanine	6	6
Proline	14-15	14-15	Lysine	15-16	15-16
Glutamic acid	15	15	Histidine	5	5
Glycine	12-15	12-15	Arginine	7	7
Alanine	12	12	Tryptophan	0	0
Valine	10-11	10-11	Glucosamine	4	4
Half cystine	16-18	16-18	Galactosamine	2	2
Methionine	5	5	A mixture of thyrotropins <i>a</i> , <i>b</i> , <i>c</i> , <i>d</i> , <i>e</i> , with <i>c</i> and <i>d</i> predominating		
Isoleucine	7	7			
			Glucosamine	5-6	
			Galactosamine	2-3	
			Mannose	4-5	
			Fucose	1 (?)	

Craig^{36,37} (see also the review by Behrens and Bromer³⁸). With thyrotropin, although further work may prove otherwise, the evidence also shows that the limit of purification may well have been reached. If this is the case, the tasks are to prepare sufficient material to complete biological testing and to investigate the physiological role of the hormone with the best preparations. From the chemist's point of view one would wish to see if biological activity can be retained after cleavage of specific covalent bonds and, as the techniques of protein and carbohydrate chemistry improve, to determine the relationships among the structures of what appear to be an interesting series of compounds and their biological activity.

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iodine), M. E. Simpson and E. S. Evans (I^{131} uptake in hypophysectomized rats), and R. W. Bates and P. G. Condliffe (I^{131} release in chicks).

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CHEMICAL DERIVATIVES OF THYROID-STIMULATING HORMONE PREPARATIONS*

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Introduction

Eleven years ago in a similar monograph published by the New York Academy of Sciences, Albert¹ stated that "most of the chemical reactions of TSH [thyrotropin, thyroid stimulating hormone] that have been reported are reactions of the hormone in impure state, and will have to be repeated when enough pure material becomes available." Unfortunately, the same situation obtains today. Indeed, some may consider it imprudent to study the reactions of a mixture of unknown composition. Notwithstanding this objection, we and others have obtained some useful information by studying the modification of TSH by various physicochemical techniques.

General Considerations

Definition. In this paper the term chemical derivative of TSH will be used for any product obtained from a TSH preparation in which there has been rupture or formation of a covalent bond, whether or not the total number of covalent bonds remains the same as in the native TSH preparation. Such derivatives will include the addition of substituent groups, the modification of covalent bonds by enzymes, and racemization. Not included in this definition will be changes in secondary or tertiary protein structure unless such changes have been induced by a procedure known to destroy a covalent bond. Other products that will be excluded are those obtained as a consequence of salt formation, polymerization, depolymerization, or binding.

Purpose. In preparing derivatives of biologically active proteins, investigators have had several goals in mind. They have attempted to correlate biological activity with some aspect of chemical structure. Such protein derivatives have also been employed in sequential and end-group analysis of proteins. In recent years radioactive protein derivatives have been used to study the fate and metabolism of various proteins.

In addition, attempts have been made to dissociate the biological activity of a protein into 2 or more component parts, so that one might be destroyed while the other is retained; for example, toxicity of toxin versus its antigenicity,² the localizing ability of a hormone preparation as opposed to its stimulatory action.³ Such procedures may introduce new biological activity; for example, inhibition instead of stimulation by hormones^{3,4} or enzymes,⁵ new antigenicity,⁶ or different physical properties; for example, tanning.⁷

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Special Problems Associated with TSH Derivatives

The foremost limitation for obtaining meaningful information about the chemical structure of TSH and its relation to biological activity is the heterogeneity of available TSH preparations. Recent reports on the purification of TSH^{8,9} suggest that many of the TSH preparations employed for the production of chemical derivatives have been less than 5 per cent pure. With a heterogeneous protein preparation it is not possible to define the product of a chemical reaction in terms of the number of substituent groups per protein molecule unless the exact composition of such a preparation is known and the reaction rates of each component are established.

Occasionally, limited conclusions may be drawn with heterogeneous preparations as to the necessity of a particular functional group for biological activity. If all the available sites of a specific functional group of a protein are blocked, and if this number corresponds with the number of substituent groups introduced, one could infer that the complete blocking of a specific functional group by a specific substituent group does or does not affect biological activity. This inference would not exclude the possibility that limited blocking of selected sites might also destroy or retain biological activity.

In this case, as well as in others where the biological activity has been modified by preparing substituted derivatives, it would be necessary to determine whether the alteration in biological activity was the direct result of blocking a functional group, of steric interference of the substituent group, or of secondary changes. These last changes would include racemization and alterations in secondary or tertiary structure.

In addition to the aforementioned chemical difficulties there remains the possibility that there may be more than one TSH, although this possibility is not supported by very many reliable data. Although there is little reason to suggest it at present, future investigations may uncover significant species differences in TSH, as have been discovered for growth hormone. If such is the case, information about the chemical structure of TSH in one species may not be directly applicable to that of another species. Finally, the difficulties of correlating chemical structure with the biological activity of TSH, whose bioassay is imprecise, deserve emphasis.

Materials and Methods

Eight different thyrotropic hormone preparations* were employed throughout the original studies reported in this paper. Biological assay of these preparations indicated that they contained approximately the thyrotropic hormone content shown in TABLE 1. All the preparations were heavily contaminated with gonadotropins, as shown by bioassay in the chick.

The various types of derivatives of these thyrotropic hormone preparations were obtained by the methods listed in TABLE 2. Within these frameworks different preparations were obtained according to the reaction conditions specified in TABLE 3. In all cases, unless otherwise specified, after completing the reaction for the stated period of time, the reaction mixture was transferred to Visking dialysis tubing and dialyzed against 5 changes of distilled water in

* Made by Parke, Davis & Company, Detroit, Mich.

the cold (4° C.) over a 2- to 5-day period. The contents of the dialysis bag were then lyophilized. Subsequently the preparation of modified hormones was assayed simultaneously for both biological stimulatory and inhibitory activity of TSH and gonadotropins.

For the evaluation of thyrotropic and gonadotropic stimulatory and inhibitory activity, 1-day-old cockerels were used. In the various experiments each animal received doses ranging between 1 and 125 mg. of the derivative preparation over a 6-day period, unless otherwise specified. During the same period of time other groups of animals were given, in addition to the derivative prepa-

TABLE 1
THYROTROPIC HORMONE CONTENT OF PREPARATIONS USED
(Expressed as U.S.P. Units per Milligram)

P.D.* 128-92	0.5	P.D. X-7057	5.0
P.D. 099861	2.5	P.D. 099802	5.0
P.D. 494 E 15	0.5	P.D. 494 E 37	2.5
P.D. X-6585	2.5	P.D. X-7774	1.0

* Parke, Davis & Company, Detroit, Mich.

TABLE 2

Reagent	Reference
Phenyl isocyanate	Hopkins & Wormall ¹⁰
Formaldehyde	Fraenkel-Conrat & Mechem ¹¹
Nitrous acid	Philpot & Small ¹²
Fluorodinitrobenzene	Sanger ¹³
Bromine	Wormall ¹⁴
Methanol	Mommaerts & Neurath ¹⁵
Ethanol	Mommaerts & Neurath ¹⁵
Sulfuric acid	Reitz <i>et al.</i> ¹⁶
Diazobenzenesulfonic acid	Sonenberg <i>et al.</i> ¹⁷
Diisopropylfluorophosphate	Balls & Jansen ¹⁸
Thioglycolic acid	Fraenkel-Conrat <i>et al.</i> ¹⁹
Iodoacetic acid	Barron & Singer ²⁰
Iodoacetamide	Fraenkel-Conrat <i>et al.</i> ²¹
<i>p</i> -Chloromercuribenzoate	Hellerman <i>et al.</i> ²²
Periodic acid	Jansen <i>et al.</i> ²³
O-Methyl isourea	Hughes <i>et al.</i> ²⁴

ration, the unmodified thyrotropic hormone preparation. Other groups of animals received only the thyrotropic hormone preparation or served as solvent-injected controls. On the seventh day each animal was injected subcutaneously with a dose ranging between 1 and 0.1 μ c. radioiodine (I^{131}). On the eighth day all were sacrificed with ether anesthesia, and the thyroids and testes were removed, dissected free of adjacent tissue, and weighed. The thyroids were placed in 5-ml. screw-cap vials containing 1 ml. of 0.1 *N* NaOH, and the radioactivity in these vials was determined in a well-type scintillation counter measured against a standard. Where intact or hypophysectomized rats, intact mice, or intact guinea pigs were employed TSH activity was estimated on the basis of increase in thyroid weight or radioiodine uptake. Gonadotropic activity was determined on the basis of increase in weight of the ovaries or ventral prostate.

TABLE 3

EFFECT OF VARIOUS REAGENTS ON STIMULATORY AND INHIBITORY ACTIVITIES OF TSH AND GONADOTROPINS

Reagent	Derivative number	Reag. conc.	Pit. prep.	Pit. prep. conc. (%)	Temp. (°C.)	Time (min.)	pH	Per cent stimulation		Per cent inhibition	
								TSH	Gon.	TSH	Gon.
Phenyl isocyanate	17	0.093 <i>M</i>	P.D. 128-92	1.0	0	60	8.0	0	0	0	0
	65	0.018 <i>M</i>	P.D. 099861	0.25	0	60	8.0	0	0	75	0
	66	0.072 <i>M</i>	P.D. 099861	0.25	0	60	8.0	0	0	75	0
		Dilution									
Formaldehyde (37%)	9*	1:10	P.D. 128-92	0.9	0	120	7.0	0	100	100	0
	21*	1:10	P.D. 128-92	0.9	0	120	7.0	0	0	0	0
	24*	1:10	P.D. 128-92	0.9	0	120	7.0	0	0	25	0
	25	1:200	P.D. 494 E 15	1.0	0	120	7.0	100	100	0	0
	26	1:30	P.D. 494 E 15	1.0	0	120	7.0	100	100	0	0
	27	1:5	P.D. 494 E 15	0.8	0	120	7.0	100	100	0	0
	28	1:2.5	P.D. 494 E 15	0.4	0	120	7.0	100	100	0	0
	29	1:10	P.D. 494 E 15	0.9	0	120	7.0	100	100	0	0
	35	Undil.	P.D. 494 E 15	1.0	0	120	7.0	100	100	0	0
	36	1:1.3	P.D. 494 E 15	1.0	0	120	7.0	100	100	0	0
	37	1:1	P.D. 494 E 15	1.0	0	120	7.0	100	100	0	0
	38	1:4	P.D. 494 E 15	1.0	0	120	7.0	100	100	0	0
	40	1:10	P.D. 128-92	0.9	0	360	7.0	100	80	0	0
	41	1:10	P.D. 494 E 15	0.9	0	360	7.0	100	100	0	0
	42	1:10	P.D. 494 E 15	0.9	0	240	7.0	100	100	0	0
	94	1:10	P.D. 494 E 15	0.9	2	360	6.8	100	100	0	0
		Reag. Conc.									
Nitrous acid	20	1.3 <i>M</i>	P.D. 128-92	0.7	0	30	4.7	0	0	0	0
	73	1.3 <i>M</i>	P.D. 099861	0.7	2	60	4.7	0	25	0	0
Fluorodinitrobenzene	92	2.5 ml./ 200 ml.	P.D. X-6585	1.0	4	1080	—	0	0	0	0
Bromine	10	10 ml./ 200 ml.	P.D. 128-92	1.0	4	360	7.4	0	0	0	50
	31	1 ml./ 200 ml.	P.D. 494 E 15	1.0	4	180	7.4	0	0	50	0

TABLE 3—(Continued)

Reagent	Derivative number	Reag. conc.	Pit. prep.	Pit. prep. conc. (%)	Temp. (°C.)	Time (min.)	pH	Per cent stimulation		Per cent inhibition	
								TSH	Gon.	TSH	Gon.
Bromine	32	7 ml./200 ml.	P.D. 494 E 15	1.0	4	360	7.4	0	0	25	0
	33	49 ml./200 ml.	P.D. 494 E 15	0.8	4	240	7.4	0	0	50	0
	69	1 ml./200 ml.	P.D. 099861	1.0	0	300	7.4	0	0	50	0
Methanol	70	7 ml./200 ml.	P.D. 099861	1.0	0	240	7.4	0	0	50	0
	14	100%	P.D. 128-92	1.0	-9	60	—	100	100	0	0
	30	100%	P.D. 494 E 15	1.0	-9	60	—	100	100	0	0
Ethanol	71	100%	P.D. 099861	1.0	2	60	—	100	100	0	0
	72	100%	P.D. 099861	1.0	2	60	—	100	100	0	0
	1*	36 N	P.D. 128-92	1.25	-30	60	—	0	0	0	75
Sulfuric acid	3	36 N	P.D. 128-92	2.50	0	60	—	0	0	0	0
	4	36 N	P.D. 128-92	2.0	-30	60	—	0	0	75	0
	5†	36 N	P.D. 128-92	1.7	0	60	—	0	0	25	25
	6§	36 N	P.D. 128-92	1.7	-30	60	—	0	0	0	50
	19†	36 N	P.D. 128-92	2.0	-9	30	—	0	0	0	0
Diazobenzene sul- fonic acid	13	0.0067 M	P.D. 128-92	0.6	0	—	9.8	0	0	0	0
Diisopropylfluoro- phosphate	22	0.32 ml./400 ml.	P.D. 128-92	0.5	2	60	8.0	100	100	0	0
	74	0.30 ml./200 ml.	P.D. 099861	0.5	2	60	8.0	100	100	0	0
Thioglycolic acid	12	0.2 M	P.D. 128-92	1.0	0	60	8.0	100	100	0	0
Iodoacetic acid	68	0.25 M	P.D. 099861	1.0	2	120	7.4	100	100	0	0
Iodoacetamide	16	0.025 M	P.D. 128-92	1.0	0	120	7.4	100	100	0	0
	67	0.25 M	P.D. 099861	1.0	2	120	7.4	0	100	75	0
p-Chloromercuriben- zoate	23	0.00025 M	P.D. 494 E 15	1.0	28	45	7.3	100	100	0	0
Periodic acid	179	0.013 M	P.D. X-7057	0.4	23	5	8.6	100	100	—	—

* Assayed in intact rat.

† Assayed in hypophysectomized rat.

‡ Assayed in mouse.

§ Assayed in guinea pig.

Results

Derivatives obtained with nonenzymatic reagents. As may be seen from TABLE 3, the addition of phenyl isocyanate to an alkaline solution of a pituitary preparation containing thyrotropic and gonadotropic activity caused the

complete destruction of these hormonal activities. In addition, such phenylureido derivatives of one of these pituitary preparations were able to reduce significantly the response to exogenously administered thyrotropin without altering the gonadotropic response. However, it may be seen (TABLE 4) that neither phenyl isocyanate alone nor phenyl isocyanate-treated bovine serum albumin modified the response of the thyroid or testes to an exogenously administered pituitary preparation.

Although the reaction of fluorodinitrobenzene with TSH and gonadotropin preparations completely inactivated these biological activities, the derivative obtained did not alter the response to exogenously administered TSH or gon-

TABLE 4
EFFECT OF PHENYL ISOCYANATE AND PHENYL ISOCYANATE-TREATED BOVINE SERUM ALBUMIN ON TSH AND GONADOTROPIC ACTIVITY IN CHICKS*

Type of treatment	Total dose (mg.)/6 days	Thyr. wt./ 100 gm. body wt.	Testes wt./ 100 gm. body wt.
BSA 19	35	12.6	53.8
+ A P†	1.5		
BSA 19‡	35	4.6	20.4
BSA 20‡	35	15.7	48.9§
+ A P	1.5		
BSA 20	35	4.7	22.2
Phenyl isocyanate	0.5 ml.	12.9	54.8
+ A P	1.5		
Phenyl isocyanate	0.5 ml.	4.1	19.4
A P	1.5	13.7§	54.3
Control	—	4.3	20.4

* The data in this and subsequent tables have been evaluated statistically according to the method of Fisher.²⁶ In all experiments the group receiving the pituitary preparation (A P) has been compared to the uninjected or solvent-injected controls (Control). The group receiving the experimental substance and pituitary preparation has been compared with the group receiving only the pituitary preparation (A P) alone. The group injected only with the experimental material was contrasted with the uninjected or solvent-injected controls (Control). The same notations for statistical significance are used throughout.

† A P is a pituitary preparation (P.D. X-6585) with TSH and gonadotropic activity.

‡ BSA 19 and BSA 20 are bovine serum albumin preparations (Armour-29534) treated with phenyl isocyanate under different conditions. The numbers are merely a code.

§ p, value between 0.01 and 0.05.

|| p, value of 0.01 or less.

adotropins. When nitrous acid was added to unmodified pituitary preparations under conditions that would produce deamination again the TSH and gonadotropin hormonal activities were destroyed without producing an inhibitor for either of these hormones.

The addition of formaldehyde to pituitary preparations with TSH and gonadotropic activity failed to give permanent destruction (TABLE 3) of these hormonal activities, nor did this procedure give us a product that could modify the response of the thyroid or testes of chicks to unmodified TSH or gonadotropins. Two different derivatives prepared from one pituitary preparation were able to inhibit thyroid response to TSH in rats. However, an ovalbumin preparation similarly treated with formaldehyde also inhibited the TSH and gonadotropic response in rats (TABLE 5).

With 3 different pituitary preparations the addition of bromine completely

inactivated TSH and gonadotropic activity. Two of these 3 brominated derivatives decreased the response to untreated TSH when administered simultaneously. On one occasion the gonadotropic response to unmodified gonadotropins was modified by the simultaneous injection of the brominated derivative, but this could not be duplicated with the brominated derivatives of the other pituitary preparations. In addition, brominated bovine serum

TABLE 5

EFFECT OF FORMALDEHYDE-TREATED OVALBUMIN ON THE THYROTROPIC AND GONADOTROPIC RESPONSE IN RATS

Type of treatment	Total dose (mg.)/ 8 days	Thyr. wt./ 100 gm. body wt.	I ¹³¹ uptake	V. P.* wt./ 100 gm. body wt.
A P†	100	8.4§	8.3	64.0§
O F 3‡	100	8.2	4.7§	48.5
O F 3	100	7.4§	5.4§	46.0§
+ A P	100			
Control	—	7.3	8.0	45.9

* V.P., ventral prostate.

† A P, pituitary preparation (P.D. 128-92) with TSH and gonadotropic activity.

‡ O F 3, derivative obtained from treating ovalbumin with formaldehyde. The number is merely a code.

§ p, 0.01 or less.

TABLE 6

EFFECT OF BROMINATED BOVINE SERUM ALBUMIN ON TSH AND GONADOTROPIC RESPONSE IN CHICKS

Type of treatment	Thyr. wt./ 100 gm. body wt.	I ¹³¹ uptake	Testes wt./ 100 gm. body wt.
A P*	16.5†	19.9†	63.3†
BSA 12‡	12.9§	14.1§	51.3§
+ A P			
BSA 12	5.7	1.8	20.9
Control	5.1	1.9	21.5

* A P, pituitary preparation (P.D. 099802) with TSH and gonadotropic activity.

† p, 0.01 or less.

‡ BSA 12, derivative obtained from treating bovine serum albumin (Armour 212-106) with bromine. The number is merely a code.

§ p, between 0.01 and 0.05.

albumin was able to modify the response to exogenously administered TSH and gonadotropins (TABLE 6).

The addition of TSH and gonadotropin preparations to anhydrous methanol or ethanol made 0.1 N with respect to HCl failed to inactivate either hormonal activity and did not produce an inhibitor for either unmodified hormone.

The reaction of concentrated sulfuric acid in the cold with a TSH and gonadotropin preparation consistently destroyed both of these hormonal activities. The resulting derivative inhibited the response to unmodified TSH both in the rat and in the chick, while the full gonadotropic response, as measured by an increase in ovarian weight, was prevented by the simultaneous injection of the derivative in the rat and mouse. However, the sulfate ester of ovalbumin

(TABLE 7) and bovine serum albumin (TABLE 8) also inhibited the TSH and gonadotropic response of rats.

When either diisopropyl-fluorophosphate, thioglycolic acid, iodoacetic acid, or parachloromercuribenzoate was added to solutions of pituitary preparations, there was no loss of TSH or gonadotropic activity, and no derivative was obtained that could inhibit the response to the unmodified hormones.

The reaction of one pituitary preparation with iodoacetamide resulted in the loss of TSH activity with little effect on gonadotropic activity. This

TABLE 7
EFFECT OF OVALBUMIN SULFATE ON TSH AND GONADOTROPIC RESPONSE IN RATS

Type of treatment	Total dose (mg.)/ 8 days	Thyr. wt./ 100 gm. body wt.	I ¹³¹ uptake	Ovary wt./ 100 gm. body wt.
A P*	100	8.6†	19.2‡	37.7‡
O S 1†	100	7.2	8.0	26.9
O S 1	100	7.5§	11.2‡	30.5§
+ A P	100			
Control		7.0	5.3	19.0

* A P, pituitary preparation (P.D. 494 E 15) with TSH and gonadotropic activity.

† O S 1, derivative obtained from treating ovalbumin with sulfuric acid. The number is merely a code.

‡ p, 0.01 or less.

§ p, between 0.01 and 0.05.

TABLE 8
EFFECT OF BOVINE SERUM ALBUMIN SULFATE ON TSH AND GONADOTROPIC RESPONSE IN RATS

Type of treatment	Total dose (mg.)/ 8 days	Thyr. wt./ 100 gm. body wt.	I ¹³¹ uptake	Ovary wt./ 100 gm. body wt.
A P*	100	10.9	45.4‡	43.3‡
BSAS 1†	111	8.0‡	18.4	24.9
BSAS 1	111	9.5§	30.4‡	30.3‡
+ A P	100			
Control		8.9	18.6	19.1

* A P, pituitary preparation (P.D. 494 E 15) with TSH and gonadotropic activity.

† BSAS 1, derivative obtained from treating bovine serum albumin (Armour 212-106) with sulfuric acid. The number is merely a code.

‡ p, 0.01 or less.

§ p, between 0.01 and 0.05.

derivative was able to antagonize the response to unmodified TSH, but not to gonadotropins.

Treatment with diazobenzene sulfonic acid yielded a derivative that was completely inactive with regard to TSH and gonadotropins and ineffective as an inhibitor of either unmodified hormone.

Periodic acid did not inactivate the thyrotropins or gonadotropins in the pituitary preparation. No assay was performed for inhibitory activity.

Derivatives obtained with enzymes. When a pituitary preparation with TSH and gonadotropic activity was incubated under the conditions specified in TABLE 9 there was complete destruction of TSH activity with both trypsin and pepsin, whereas chymotrypsin caused about 25 per cent destruction under the

same conditions as used for trypsin. Pepsin also destroyed gonadotropic activity completely, but trypsin destroyed approximately 50 per cent of the gonadotropic activity under conditions that completely inactivated TSH. Chymotrypsin, on the other hand, destroyed approximately 25 per cent of the TSH or gonadotropic activity under conditions where trypsin destroyed all of the TSH activity and one half of the gonadotropic activity. Neither carboxypeptidase nor papain had any effect on TSH or gonadotropic activity under the conditions specified in TABLE 9.

Studies with Acetylated Thyrotropin

Mechanism of inhibition. We previously have reported studies³ with acetylated thyrotropin that indicated that this derivative was an effective inhibitor

TABLE 9
EFFECT OF PROTEOLYTIC ENZYMES ON TSH AND GONADOTROPIC ACTIVITY

Enzyme	Enzyme conc. (mg. %)	A P* conc. (mg. %)	Time (hours)	pH	Temp. (°C.)	Medium	Per cent biological activity	
							TSH	Gonad.
Carboxypeptidase	0.1 ml.	100	3	8.0	37	0.05 M Borate buffer	100	100
Trypsin	10	100	3	7.85	37	0.2 M PO ₄ buffer	0†	50‡
Chymotrypsin	10	100	3	7.85	37	0.2 M PO ₄ buffer	75‡	75‡
Pepsin	10	100	3	2.0	37	0.01 M HCl	0†	0†
Papain	10	100	3	6.47	37	0.05 M Citrate buffer	100	100

* A P, pituitary preparation (P.D. X-7057) with TSH and gonadotropic activity. The biological activity was determined in a 3-day bioassay.

† p, 0.01 or less.

‡ p, between 0.01 and 0.05.

of TSH, whether exogenously administered or endogenously produced. Indeed, such preparations were found^{26,27} to be useful clinically in promoting improvement in hyperthyroidism and thyroid cancer. Originally we suggested^{3,27} that this nonstimulatory derivative antagonized the action of TSH by localizing in the thyroid and blocking the localization and stimulation of native TSH, that is, a form of "competitive inhibition." Several other possibilities, however, have been considered that might account for the inhibition of thyroid function following the administration of acetylated TSH preparation.

It seems unlikely that inhibition with acetylated TSH is of a nonspecific type due possibly to the acid nature of acetyl derivatives of proteins or to some special property of *N*-acetyl amino acids, or the acetyl group alone. It may be seen (TABLE 10) that no other acetylated pituitary protein, milk protein, or serum protein tested was able to inhibit the thyroid response to TSH, nor were any of 10 *N*-acetyl amino acids, glucose pentaacetate, or acetic acid alone (TABLE 11) effective inhibitors of TSH. It would thus appear that the

derivative obtained from acetylation of a pituitary preparation that has TSH and gonadotropic activity is a specific acetyl compound with regard to its ability to inhibit TSH activity.

Is it possible that the noninactivated gonadotropins remaining in the pituitary preparation act in some fashion to inhibit thyroid function? This seems unlikely in view of the fact that inhibition of TSH activity has been obtained with acetylated pituitary preparations in which all of the gonadotropic activity has been destroyed.

We have considered the possibility that the mechanism of inhibition of TSH activity by acetylated TSH preparations is not through a peripheral antagonism of TSH, but through some form of action on the pituitary. This might occur as a result of the secretion of another pituitary hormone that antago-

TABLE 10
ACETYLATED PROTEINS NONINHIBITORY FOR TSH

Acetylated bovine serum albumin
Acetylated bovine growth hormone
Acetylated lactalbumin
Acetylated casein

TABLE 11
ACETYL COMPOUNDS NONINHIBITORY FOR TSH

Acetyl-D,L alanine
Acetyl-L tyrosine
Acetyl-D,L phenylalanine
Acetyl-L leucine
Acetyl-L tryptophan
Acetyl-D,L valine
Acetyl glycine
Acetyl-L glutamic acid
Acetyl-D,L methionine
Acetyl-L phenylalanine
D α glucose pentaacetate
Acetic acid

nized TSH activity or through the inhibition of secretion of TSH by the pituitary. These possibilities seem highly unlikely in view of the fact that newborn chicks in which these derivatives have been tested have thyroids that show little histological evidence of stimulation²⁸ and thus presumably are not being exposed to TSH. This inference is consistent with the observation that acetylated TSH alone has little effect on thyroid function in newly hatched chicks.³ It is only with the administration of exogenous TSH and its associated thyroid stimulation that the inhibition by acetylated TSH may be demonstrated. Finally, the inhibitory action of acetylated TSH for administered TSH in the hypophysectomized human (FIGURE 1) has been demonstrated.

The question has been raised that the inhibition of TSH activity with acetylated TSH preparations may be the consequence of antihormone formation, and possibly similar doses of unmodified TSH would have an inhibitory action. We have tested the latter possibility and have noted (TABLE 12) that not only

do large doses of unmodified TSH not inhibit thyroid function but, as expected, they promote marked stimulation of the thyroid. Although the sera of chicks (TABLE 13) injected with either TSH, acetylated TSH, or both pre-

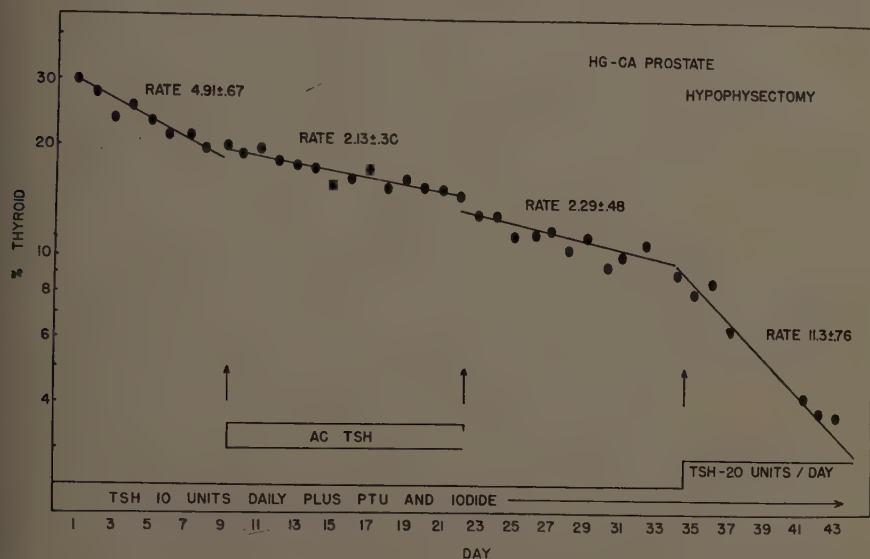


FIGURE 1. Effect of an acetylated pituitary preparation on the decay rate of radioiodine in the thyroid of a hypophysectomized patient receiving TSH. Throughout the experiment the patient received propyl thiouracil (200 mg. every 8 hours) and saturated potassium iodide (1 gm./day). He received TSH intramuscularly twice daily in the daily dose indicated and acetylated TSH intramuscularly (60 mg. twice daily). The data have been plotted semi-logarithmically so that the slope of the I^{131} remaining in the gland represents the rate of loss of iodine from the thyroid. These fractional rates of loss and standard errors (in per cent per day) were calculated by the method of Kenney and Keeping.²⁹ This patient was studied with Richard S. Benua.

TABLE 12
EFFECT OF GRADED DOSES OF A PITUITARY PREPARATION
ON TSH AND GONADOTROPIC ACTIVITY

Dose of A P* mg./chick/ 6 days	Thyr. wt./ 100 gm. body wt.	I^{131} uptake	Testes wt./ 100 gm. body wt.
0.25	8.4†	7.4†	34.3†
1.25	12.6†	13.3†	47.6†
25	22.4†	16.7†	81.0†
100	24.4†	13.5†	95.0†
Control	3.7	0.9	15.6

* A P, pituitary preparation (P.D. X-7057) with TSH and gonadotropic activity.

† p, 0.01 or less.

vented the full response to TSH of recipient chicks given unmodified TSH as well, one cannot be certain that this represented antihormone formation. Perhaps TSH or acetylated TSH elicits some proteolytic enzyme or another type of TSH inhibitor in the serum of chicks. The sera of patients treated with acetylated TSH were not able to modify the response of chick thyroids

to unmodified TSH (TABLE 14). Finally, the fact that we have observed inhibition of thyroid function within hours in man (FIGURE 1) or chicks²⁷ after a single injection of acetylated TSH makes it unlikely that the mode of action is through antihormone formation.

TABLE 13
ASSAY FOR ANTIHORMONES IN CHICKS TREATED WITH AN
ACETYLATED PITUITARY PREPARATION

Donor chicks				Recipient chicks				
Type of treatment	Total dose of acetyl A P (mg.)	Total dose of A P	No. of days of treat.	Total dose		Thyr. wt./100 gm. body wt.	I ₁₃₁ uptake	Testes wt./100 gm. body wt.
				Sera (cc.)	A P (mg.)			
T I 149*	50	—	6	5	0.5	8.0†	5.9	42.3
				5	—	4.2†	0.5	25.5†
T I 149 + A P†	50	10	6	5	0.5	8.8§	5.5	46.6
				5	—	6.5†	0.5	19.0
A P	—	10	6	5	0.5	9.3§	2.9	49.2
				2.5	—	7.7	0.5	15.5
No. treat.	—	—	—	5	0.5	11.2§	5.9	47.7
				4	—	7.9	0.5	16.9
				—	(Incub.)			
				—	0.5	11.6	5.0§	41.8†
				—	0.5	14.3	3.7	47.6†
				Control		9.1	3.2	13.4

* T I 149, acetylated derivative obtained from pituitary preparation (P.D.X-7057). The number is merely a code. The sera were incubated with the pituitary preparation for a week and a three-day bioassay was employed.

† A P, pituitary preparation (P.D. X-7057) with TSH and gonadotropic activity.

‡ p, 0.01 or less.

§ p, between 0.01 and 0.05.

TABLE 14
ASSAY FOR ANTIHORMONES IN PATIENTS TREATED WITH
ACETYLATED PITUITARY PREPARATIONS*

Donor patients				Recipient chicks			
Patient	Diagnosis	Time after onset of acetyl TSH R (days)	Total dose of acetyl TSH (mg.)	Total dose		Thyr. wt. (mg.)	Testes wt. (mg.)
				Sera (cc.)	A P† (mg.)		
C.K.	Graves' disease	31	1515	9	1	6.3	25.9
M.W.	Follic. Ca. of thyroid	35	1947	8	1	6.9	28.1
S.R.	Follic. Ca. of thyroid	Pre-R	—	4	1	5.3	27.4
		42	3290	10	1	5.5	30.3
J.C.	(1) Follic. Ca. of thyroid	Pre-R	—	3.5	1	6.0	23.4
		Pre-R	—	3	1	5.3	22.2
	(2) Hyperthyroidism	53	3350	10	1	6.8	23.6
				Control	1	5.7†	24.8†
						2.7	5.9

* Studied with our colleague Jacob L. Brener.

† A P, pituitary preparation (P.D. X-7057) with TSH and gonadotropic activity. The sera were incubated with the pituitary preparation for one week, and a 3-day bioassay was employed.

‡ p, 0.01 or less.

Conditions for the preparation and assay of acetylated TSH. After some initial success^{3,4,26,27} in the use of acetylated TSH preparations as inhibitors of thyroid function, it was observed that not all acetyl derivatives obtained from the same original TSH preparation and made under apparently similar reaction conditions gave equivalent inhibition. We therefore investigated the various conditions that might influence the nature of the acetylated derivative. In general, it was possible to acetylate TSH preparations under a variety of conditions (TABLE 15) and obtain an acetylated TSH preparation that was inhibitory for TSH. Thus, acetylation in anhydrous acetic anhydride, distilled water, or sodium acetate solutions at pHs between 7.0 and 9.0 yielded a

TABLE 15
CONDITIONS UNDER WHICH INHIBITORY ACETYLATED PITUITARY
PREPARATIONS HAVE BEEN PREPARED

A. Medium
(1) Anhydrous acetic anhydride
(2) Distilled water
(3) 0.1 M sodium acetate
(4) 0.5 M sodium acetate
B. pH in aqueous media
(1) 7.0
(2) 8.15
(3) 9.0
C. Temperature
0–31.0° C.
D. Duration
1–360 min.
E. Acetylating agent in aqueous media
(1) Acetic anhydride—1 ml./gm. protein
(2) Acetic anhydride—5 ml./gm. protein
(3) Acetic anhydride—10 ml./gm. protein
F. Protein concentration
(1) 0.25%
(2) 0.50%
(3) 1.00%
G. Recovery
(1) Dialysis and freeze-drying
(2) Isoelectric (?) precipitation at pH 4.1
(3) Precipitation in 90% cold acetone
(4) Precipitation in 85% cold ethanol
a. precipitate
b. supernatant

product that was an inhibitor of TSH. In aqueous media an inhibitory product was obtained when acetic anhydride was added in amounts between 1 ml./gm. protein to 10 ml./gm. protein. In addition, the temperature could be varied between 0° C. and 31° C., the protein concentration between 0.25 and 1.00 per cent, and the duration of the reaction between 1 and 360 min. An inhibitory acetylated TSH derivative was prepared from sheep, hog, and beef pituitary preparations. Recovery of the inhibitory product could be effected by dialysis in the cold, followed by freeze-drying, isoelectric (?) precipitation at pH 4.1, precipitation in 90 per cent cold acetone or precipitation in 85 per cent cold ethanol. In the latter case the inhibitor was divided between the precipitate and the supernatant.

Guanidination failed to inactivate the TSH and gonadotropic activities

of our pituitary preparation (TABLE 16) and failed to produce an inhibitor for these hormonal activities. Acetylation of the unmodified pituitary preparation or the guanidinated derivative destroyed the TSH activity and gave an acetylated derivative that was a TSH inhibitor (TABLE 16). Acetylation of the guanidinated derivative failed to inactivate the gonadotropins, while acetylation of the unmodified pituitary preparation partially inactivated the gonadotropins (TABLE 16). Under no circumstances studied (TABLE 16) was an inhibitor of gonadotropins found.

With such wide latitude in the reaction conditions apparently available for the preparation of inhibitory acetylated TSH, it appeared that the erratic responses may have been intrinsic in the bioassay. The injection schedule of the bioassay previously employed³ was varied, so that either the same daily

TABLE 16
EFFECT OF GUANIDINATION AND ACETYLATION ON THE STIMULATORY AND
INHIBITORY ACTIVITIES OF ACETYLATED PITUITARY PREPARATIONS

Prep.	Derivative	Type of treatment	Total dose mg./chick /6 day	Thyr. wt./100 gm. body wt.	I ¹³¹ I uptake	Testes wt./100 gm. body wt.
T I 175*	Guanidinated	T I 175	87	21.5	19.3	84.9†
		+ A P†	1			
T I 176	Guanidinated + acetylated	T I 175	87	26.5§	19.5§	113.4§
		T I 176	87	13.8	14.3	64.7
		+ A P	1			
T I 178	Acetylated	T I 176	87	7.1	6.1§	49.0§
		T I 178	88			
		+ A P	1	10.5§	10.1†	67.4†
		T I 178	88	4.7	1.2§	35.7§
		A P	1	18.8§	13.9§	52.6§
		Control		5.8	1.0	18.0

* T I 175, 176, and 178, derivatives, as indicated, of a pituitary preparation (P.D. X-7057). The numbers are merely a code.

† A P, pituitary preparation (P.D. X-7057) with TSH and gonadotropic activity.

‡ p, between 0.01 and 0.05.

§ p, 0.01 or less.

dose or the same total dose was administered for periods lasting 6 to 20 days. It appeared (TABLE 17) that injection periods of 13 or 20 days were more satisfactory than those of 6 days in demonstrating inhibition of thyroid growth and function with acetylated TSH. It was also necessary to maintain the same daily dosage rather than the same total dosage in order to demonstrate significant TSH inhibition with acetylated TSH.

Using a 13-day bioassay with a large total dose and a ratio of acetylated TSH to unmodified TSH of 75:1, we examined the inhibitory response of 6 preparations of acetylated TSH. These preparations were maintained in the dry state in the cold (4° C.) until bioassayed. As may be seen from TABLE 18, the TSH inhibitory response in these chicks was extremely erratic. In addition, it may be observed (TABLE 18) that identical doses of the same lot of pituitary preparation injected into 5 different batches of chicks of the same age gave increases in thyroid weight over the untreated controls that varied

TABLE 17

EFFECT OF DURATION OF INJECTIONS ON THE BIOASSAY OF THE INHIBITORY ACTIVITY OF AN ACETYLATED PITUITARY PREPARATION

Type of treatment	Total dose (mg.)	Days of inject.	Thyr. wt./100 gm. body wt.	I ¹³¹ uptake	Testes wt./100 gm. body wt.
T I 248*	75	6	12.6	7.7	50.9
+ A P†	1				
A P	1	6	11.7‡	8.0‡	48.1‡
Control	—	6	7.1	1.3	18.5
T I 248	75	13	8.3	4.8	25.5
+ A P	1				
T I 248	150	13	10.4§	5.7§	37.5
+ A P	2				
A P	1	13	8.2	5.2	29.2
A P	2	13	13.0‡	8.9‡	29.2
Control	—	13	6.6	3.7	30.8
T I 248	75	20	6.0	4.5	35.4
+ A P	1				
T I 248	225	20	8.7§	5.4	31.9
+ A P	3				
A P	1	20	7.0	5.0	29.4‡
A P	3	20	10.2‡	7.2§	29.4
Control	—	20	6.0	3.7	21.7

* T I 248, acetylated derivative obtained from pituitary preparation (P.D. X-7774). The number is merely a code.

† A P, pituitary preparation (P.D. 494 E 37) with TSH and gonadotropic activity.

‡ p, 0.01 or less.

§ p, between 0.01 and 0.05.

TABLE 18

EFFECT OF REPEATED ASSAYS FOR TSH-INHIBITORY ACTIVITY* ON THE SAME LOTS OF ACETYLATED† PITUITARY PREPARATIONS

Lot	Inhibition				
	4/29/59	5/6/59	5/13/59	5/20/59	5/27/59
T I 242	0	75§	0		
T I 243 E	0	0	0		
T I 243 E 2	0	25§	100§		
T I 244		0	0	50	
T I 245 E			0	75§	75§
T I 245 E 2			100	75	50§
Per cent stimulation‡ A P	62	86	33	53	17

* Inhibition is indicated as the per cent decrease in TSH response (thyr. wt./100 gm. body wt. and I¹³¹ uptake) toward control values.

† All acetylated derivatives were obtained from the same unmodified pituitary preparation (P.D. X-7774). The numbers merely represent a code.

‡ Per cent stimulation represents the proportional increase in TSH end points (thyr. wt./100 gm. body wt.). The same pituitary preparation (P.D. 494 E 37) was used for each of the 5 groups of chicks.

§ p, between 0.01 and 0.05.

|| p, 0.01 or less.

from 17 to 86 per cent. It appeared that the variability in the inhibitory response was due not so much to the variable product obtained from an inadequately controlled reaction as from some indeterminates in the bioassay.

Discussion

Without the advantages of a homogeneous thyrotropin preparation and precise analyses for substituent group introduced and the functional group or groups that have reacted, as well as without knowledge of the changes in secondary or tertiary structure of the protein that may have been induced, it is hazardous to correlate biological activity of thyrotropin with some aspect of chemical structure. Within this framework, however, there are suggestive data that bear emphasis.

In general, it would appear that reagents that react with the amino groups of proteins, even though these are not the only sites of reaction, destroy the biological activity of thyrotropin and gonadotropin. Of course, incomplete reaction of the reagent with the amino group, such as would occur with adverse stoichiometry, or other reaction conditions may result in partial destruction of these biological activities, as has been demonstrated with acetylation³ and deamination with nitrous acid (TABLE 3). The reaction of the protein with acetic anhydride,³ phenyl isocyanate, or fluorodinitrobenzene, reagents that introduce substituent groups into the protein, led to the destruction of these hormonal activities. When the amino group was removed by deamination with nitrous acid there was also destruction of the biological activity.

Since the above reagents may react with either the alpha or epsilon amino groups, a reagent was sought that reacted with only one of these types of amino groups. *O*-methyl isourea reacts only with the epsilon amino group of lysine to give a guanidinated derivative, homoarginine.²⁴ The latter contains a free guanidino group that may be positively charged at the appropriate *pH*. Guanidination of pituitary preparations with TSH and gonadotropic activity had no significant effect on the biological activity of these hormones (TABLE 16). It would thus appear that the epsilon amino group of lysine is not essential for these biological activities. In order to maintain biological activity it is not sufficient merely to maintain a charge on the guanidino group, for guanidination followed by acetylation is followed by destruction of biological activity, as occurs after acetylation alone. The loss of biological activity after guanidination and acetylation suggests that the alpha amino groups are essential for biological activity. If these data are taken to mean that the alpha group is essential to biological activity, TSH would differ from 2 other pituitary hormones, growth hormone^{30,31} and prolactin.³¹

The inactivation of the TSH and gonadotropic activity of 3 different pituitary preparations by bromination is not surprising in view of the previously reported inactivation of TSH by iodine,^{1,32-34} bromine,³⁴ and other oxidizing agents.^{1,32} The preparations in the present study were very likely brominated in solutions of *pH* 7.4. The destruction of thyroid- and gonad-stimulating ability by bromine could result from either oxidation of sulfhydryl groups, bromination of tyrosine residues, or both. However, *p*-chloromercuribenzoate²² and iodoacetic acid,²¹ 2 reagents reported to react with sulfhydryl groups, did not inactivate TSH and gonadotropins. Iodoacetamide, which

also reacts with this same functional group, did inactivate TSH and had little effect on gonadotropins. It may be that *p*-chloromercuribenzoate and iodoacetic acid act reversibly on proteins, and perhaps this effect may account for the presence of biological activity in pituitary preparations treated with these reagents. It may be that the introduction of a charged group such as acetate or mercuribenzoate will preserve TSH and gonadotropic activity, while an uncharged substituent group such as acetamide will destroy thyrotropic activity, as we have found. It would appear that the sulfhydryl groups are essential for thyrotropic activity, since the addition of oxidizing agents to such protein preparations leads to a reduction in biological activity.³²⁻³⁴ In fact, some of the thyrotropic activity of pituitary preparations treated with iodine,^{1,32,35} or bromine³⁴ could be recovered by the subsequent treatment with reducing agents. The essential role of sulfhydryl groups for thyrotropic activity is emphasized by the observations^{1,34,36} that unmodified pituitary preparations may have the activity of TSH augmented by treatment with reducing agents. This augmentation also suggests that not all of the sulfur groups in the protein are present as sulfhydryl groups, but that some may be converted to such groups. In this regard, it is difficult to reconcile the augmentation effect with the observation³⁷ that the addition of cysteine to pituitary preparations will destroy rather than augment thyrotropic activity. It may be that under certain conditions the disulfide link in cystine is reduced by some reducing agents, and this cystine is crucial for biological activity.

If the loss of biological activity with bromination is the consequence of ortho substitution on the benzene ring of tyrosine, this effect would be consistent with the loss of activity noted with iodination,^{1,32-34} bromination,³⁴ and diazotization^{17,38} (TABLE 3). It was previously observed^{17,38} that extensive diazotization was necessary to destroy TSH activity completely. Six diazo groups per mole of TSH (assumed molecular weight of 10,000) were required for complete TSH inactivation.

Esterification of carboxyl groups with acidic ethanol or methanol failed to inactivate the thyrotropic and gonadotropic activity. This observation is consistent with the lack of effect of carboxypeptidase (TABLE 9) on these hormonal activities. Since the latter acts on the terminal peptide bond adjacent to the C-terminal residue and esterification with anhydrous alcohols acts on all available carboxy groups, it would appear that no free carboxyl groups are necessary for biological activity.

Although concentrated sulfuric acid reacts with the aliphatic hydroxyl groups of serine and threonine in proteins to give sulfate esters, it is very likely that other reactions also occur, such as with sulfhydryl groups and phenolic hydroxyl groups, as well as with benzene rings. For this reason it is difficult to determine the significance of the destruction of TSH and gonadotropic activity by concentrated sulfuric acid. Although this is a drastic procedure for a protein, it is interesting that such treatment to give insulin sulfate does not destroy biological activity.¹⁶

The lack of effect of thioglycolic acid on TSH and gonadotropic activity suggests that, if these hormones have disulfide bonds, they may not be available for reduction by thioglycolic acid or, if they have been reduced, they may not be crucial for biological activity. As mentioned previously, it has

been observed³⁷ that cysteine reduced the TSH activity of pituitary preparations. Perhaps some reagents are more effective than others in reducing disulfide residues in proteins.

The absence of an effect of periodic acid on TSH or gonadotropic activity suggests that these hormones do not have a terminal hydroxy amino acid.^{39,40} If carbohydrate was present in this reputed^{37,41} glycoprotein and if it reacted with periodic acid,⁴² it would suggest that the carbohydrate is not crucial for the biological activity of TSH. It should be emphasized that under very similar conditions others⁴³ have found that periodic acid destroyed 85 to 90 per cent of TSH and FSH activity, with marked destruction of ICSH activity. It is interesting that under the conditions tested⁴³ there was not complete destruction of TSH, FSH, or ICSH activity.

Of those derivatives that were inactive with respect to TSH or gonadotropic activity, several were able to inhibit the response to unmodified TSH. Acetylated TSH preparations appear to be specific inhibitors of TSH activity.³ No other acetylated protein (TABLE 10), acetylated amino acid (TABLE 11), or acetic acid (TABLE 11) has this inhibitory property. Similarly, some phenylureido derivatives of TSH preparations were able to inhibit the thyroid response to unmodified TSH (TABLE 3), while phenylureido bovine serum albumin (TABLE 4) or phenyl isocyanate alone (TABLE 4) did not have this effect. Brominated pituitary preparations also had some inhibitory activity against TSH (TABLE 3), as did brominated bovine serum albumin (TABLE 6). This was the case also with the sulfate ester of a TSH preparation (TABLE 3), ovalbumin sulfate (TABLE 7), and bovine serum albumin sulfate (TABLE 8). This suggested that this type of inhibition was nonspecific. A similar circumstance was observed with formaldehyde-treated pituitary preparations (TABLE 3) and formaldehyde-treated ovalbumin (TABLE 5).

Not all inactivated derivatives that have had their amino groups modified were inhibitory for unmodified TSH. Fluorodinitrobenzene and nitrous acid destroyed the TSH and gonadotropic activities without producing an inhibitor. In addition, the diazotized pituitary preparation with inactivated TSH and gonadotropins was unable to inhibit the response of these unmodified hormones. It may be that inactivated TSH preparations that are not specific inhibitors for unmodified TSH have had both the localizing and stimulatory portions of the molecule destroyed.³ The significance of the inhibition of TSH activity with an iodoacetamide-inactivated pituitary preparation is difficult to evaluate, since similarly reacted control proteins have not been tested for inhibitory activity.

The results reported herein (TABLE 9) on the effect of proteolytic enzymes on TSH and gonadotropic activity are essentially consistent with earlier studies.⁴⁴⁻⁴⁶ In the present study chymotrypsin was less effective than trypsin in destroying TSH or gonadotropic activity, which varies with what was previously reported.⁴⁴ Although Chow *et al.*⁴⁴ reported complete destruction of TSH with chymotrypsin after 12 to 75 per cent digestion and with trypsin after 18 to 80 per cent digestion, it is difficult to evaluate the significance of the degree of digestion. It may be that the bulk of the digested material is derived from some of the contaminants in a heterogenous pituitary preparation. Under such circumstances the degree of digestion of the specific protein,

thyrotropin, may be greater or less than the degree represented. The degree of digestion reported⁴⁴ is, in effect, only an average number. The same considerations apply to the effect of proteolytic enzymes on gonadotropic activity. Although we noted (TABLE 9) no destruction of TSH or gonadotropins with papain, Chow *et al.* reported⁴⁴ some destruction of the latter without effect on TSH when the digestion was carried to 65 per cent. Perhaps this represents some difference in reaction conditions. In our studies carboxypeptidase had little effect on TSH or gonadotropins, whereas Chow *et al.*⁴⁴ observed no effect on TSH with some destruction of gonadotropic activity. The latter was attributed by these authors to contaminating trypsin.

In considering the conditions that may be employed to obtain acetylated TSH preparations that are inhibitory for TSH, it would appear that conditions that permit the complete acetylation of all the free amino groups³ are adequate for the preparation of an inhibitor. We have not investigated the possibility that incompletely acetylated pituitary preparations might be equally inhibitory for TSH. In this regard we have noted³ that acetylation for shorter reaction times in the cold failed to inactivate completely all the TSH in a pituitary preparation. The derivative is still nondialyzable after acetylation and, to some extent, it is soluble in 85 per cent ethanol.

Although the erratic inhibitory response of acetylated TSH seemed to reside in the bioassay, prolongation of the assay period did not completely eliminate the variable response. Neither the solvent used for injections nor the pH of these solutions affected inhibitory response, since water, saline, and alkali have been employed with pHs between 4.2 and 9.8 without different responses. Incubation of the acetylated pituitary preparations in alkali prior to injections did not enhance or decrease the inhibitory activity. The site of injection did not influence the inhibitory response to acetylated TSH. It is difficult to account for the factors that might permit one batch of chicks to demonstrate an inhibitory response to acetylated TSH, while a second batch of chicks shows no inhibition to the same lot of acetylated TSH.

Until now we have found little species specificity for the stimulatory activity of TSH preparations or the inhibitory activity of acetylated pituitary preparations. The latter derivative, obtained by acetylation of sheep, beef, or hog preparations, was able to inhibit the TSH response in chicks or humans.

Conclusion

Despite the known heterogeneity of the pituitary preparations we have employed in these studies, we have gained some insight into the conditions that affect TSH activity. In addition, some suggestive data have been obtained concerning the portions of the hormone molecule responsible for biological activity. Of course, conclusive evidence will not be forthcoming until a completely homogeneous TSH preparation is available. Within this framework it would appear that the portions of the molecule crucial for biological activity are the alpha amino group, aliphatic hydroxyl groups, sulfhydryl groups, and tyrosine residues. Those groups that seem unnecessary for biological activity, if they are present, are the carboxyl, epsilon amino, and disulfide groups, as well as glucose residues, terminal hydroxyamino acids, and C-terminal amino acids.

Some inactivated thyrotropic hormone preparations can inhibit the thyroid response to unmodified TSH preparations. In the inhibitory group are found the acetyl derivative as well as the phenylureido derivative of pituitary preparations, which give moderately specific inhibition. Other inactivated TSH preparations, after treatment with bromine, concentrated sulfuric acid, or formaldehyde, also modified the thyroid response to exogenous TSH. This inhibition was considered to be nonspecific inasmuch as similarly treated control proteins demonstrated inhibition as well. Some inactivated preparations were noninhibitory for exogenously administered TSH.

A detailed study of the conditions of preparation of inhibitory acetylated TSH revealed wide latitude in these conditions. The erratic response in laboratory animals for TSH inhibitory activity appears to be due to some as-yet-undefined variables in the bioassay. The most likely mechanism for the inhibition of TSH activity with nonstimulatory TSH preparations remains one of "competitive inhibition."

Acetylated thyrotropin has been used to inhibit thyroid growth and function in patients with thyroid cancer and hyperthyroidism,²⁶ and in individuals without thyroid disease.

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REGULATION OF TSH RELEASE

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The functional activity of many biological systems is regulated by more than one mechanism. Some of these mechanisms are in the form of servo-controlled systems, and others are specifically designed to alter the activity level of the system. Each mechanism is composed of a number of sequential steps involving different anatomic and functional units. These mechanisms are inter-related either at the final active tissue cell or at one of the intermediate steps of the several mechanisms.

The analysis of such controlled systems involves an anatomic and physiological mapping of the several steps of each mechanism and of the interrelationships among them. Such studies are a prerequisite to the more detailed investigations of each step in terms of biochemical mechanisms.

We shall describe and criticize the attempts to analyze the mechanisms regulating thyroid stimulating hormone (TSH) release from the pars distalis. No attempt will be made to review the literature, as most of the original references will be found in recent reviews and publications.^{4,11,26,27,32,33,36}

The main difficulties in the design and interpretation of experiments dealing with TSH release may be listed. One consists of determining which component of the several mechanisms is the primary site of action of an administered chemical. The close proximity between the hypothalamus and pituitary increases this difficulty. Another is determining the primary effect of ablation or stimulation of one component. A third is that the anatomic limits of certain components are not known: for example, the "thyrotrophin area of the hypothalamus." It is therefore impossible to be certain that hypothalamic lesions have removed all hypothalamic influence on the pituitary. Similarly, it is not yet known whether interruption of the portal circulation hypophysectomized by stalk section or pituitary grafts completely eliminates all neural influence on the pituitary. Finally, it should be noted that a mechanism that is studied and found to be operating over a short experimental period may not be identical with the normal long-term regulatory mechanism operating during the day.

GENERAL METHOD

We have tried to overcome some of these difficulties by using a new technique of prolonged and continuous microinjection of chemicals subcutaneously or directly into various sites in the unanesthetized unrestrained rat. The microinjector and the operative and other details of some of these techniques have been described.^{47,48} Recent additions and modifications will be published soon, but a brief description of the general methodology is given in this paper.

A microinjector has been devised that utilizes the osmotic pressure of a saturated solution of Congo red as the motive force. Basically the injector consists of three compartments. A nonrigid water compartment is separated from the Congo red compartment by a semipermeable cellophane membrane. Water moves by osmosis into the Congo red compartment, and this squeezes the drug out of a third compartment.

The injector can be buried subcutaneously in the rat or attached permanently to the tail in such a way that there is very little disturbance to the animal.

The injector can deliver drugs constantly and continuously in a volume range of 0.02 ml. to 2.0 ml. per day for many days or weeks. It can be started or stopped, the rate of delivery can be measured and varied, and the drug being delivered can be changed in any experimental period without disturbing the animal.



FIGURE 1. Rat with tube fixed in hypophyseal fossa and connected to subcutaneously placed injector.



FIGURE 2. Rat with microinjector attached to tail. Tube passes subcutaneously up tail to site of injection.

The drug is delivered through a fine polyethylene tube (50 to 200 μ diameter) that runs subcutaneously to the site of interest. For studies of TSH release it is advantageous to be able to administer the drug either subcutaneously or directly and without systemic effect to the pituitary *in situ*, to the pituitary grafted in the anterior chamber of the eye, or under or into the hypothalamus.

FIGURE 1 shows a rat with a tube fixed in the hypophyseal fossa and connected to a subcutaneously placed injector. FIGURE 2 shows a recent modification of the technique whereby the injector is attached to the tail of the rat, the tube passing subcutaneously up the tail to the site of injection. FIGURE 3 shows a polyethylene tube (containing India ink for display) passing through

the ventrocephalic edge of the hypophyseal fossa. FIGURE 4 is a photomicrograph showing the relation of the tube to the pituitary. FIGURE 5 is a photomicrograph of a mid-line sagittal section through the hypothalamus, pituitary, and base of the skull after the vascular system has been perfused with India ink. The portal vessels pass around the plastic tube to reach the pars distalis. The



FIGURE 3. Polyethylene tube (proximal part containing India ink for display) passing through ventrocephalic edge of hypophyseal fossa. A, polyethylene tube; T, trigeminal nerve; HF, hypophyseal fossa.

connective tissue surrounding the tube is supplied by these vessels, and this may assist in allowing a local, continuous, small application of a drug to influence the entire pars distalis. FIGURE 6 shows a pituitary graft growing in the anterior chamber of the eye (*top*) and a polyethylene tube (filled with India ink) fixed in the anterior chamber (*bottom*). Drugs administered through this tube then will diffuse from the aqueous solution to the graft.

Continuous direct application of chemicals to a particular tissue often can

achieve a primary physiological or pharmacological effect without significant primary systemic effects. Such studies therefore can be used to determine the primary site of action of drugs or hormones without the complication of primary systemic effects. Giving the drug continuously minimizes difficulties in interpreting the specificity of action of a chemical that may have an activat-



FIGURE 4. Photomicrograph of horizontal section through left half of hypophysis, showing connective tissue reaction and position of tube in relation to pituitary. PD, pars distalis; PI, pars intermedia; PN, pars nervosa; CT, connective tissue around tube. $\times 70$.

ing or inhibiting effect on the tissue. Moreover, it is thus demonstrated that the mechanism being studied can act continuously over a prolonged period.

Continuous systemic administration of hormones is often of value in that such administration more closely approximates natural hormone secretion by a gland and therefore may be used to study physiological replacement after endocrine ablation. FIGURE 7 shows the effects of various doses of L-thyroxine on the thyroidal I^{131} release rate (TRR) as determined by *in vivo* counts over the

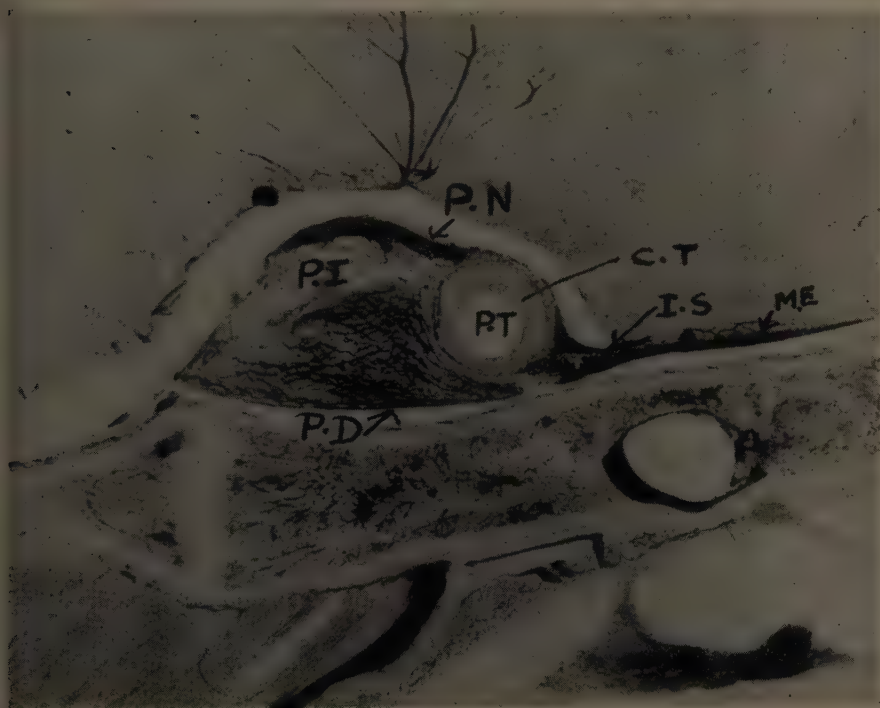


FIGURE 5. Photomicrograph of midline sagittal section through hypothalamus, pituitary, and base of skull, with vascular system perfused with India ink. The tube had been implanted in pituitary for 8 days. PN, pars nervosa; PI, pars intermedia; PD, pars distalis; IS, infundibular stem; ME, median eminence; PT, plastic tube; CT, connective tissue. $\times 8.5$.

neck (*left*), and on the thyroid weights (TW) obtained post-mortem (*right*), in rats that had been receiving daily subcutaneous injections of 20 mg. propylthiouracil (PTU) in suspension for 10 days. It is evident that continuous administration of L-thyroxine is more effective in preventing goiter and depressing the TRR than the same dose given in single daily injections. The thyroxine in both experiments was made up and delivered in 0.2 ml. of M/250 glycine buffer at pH 9.5. Under the conditions of this experiment 0.7 to 0.8 $\mu\text{g.}$ of L-thyroxine/100 gm./day by continuous injection is equivalent to 1.5 $\mu\text{g.}$ of L-thyroxine by single daily injection, and these are the doses necessary

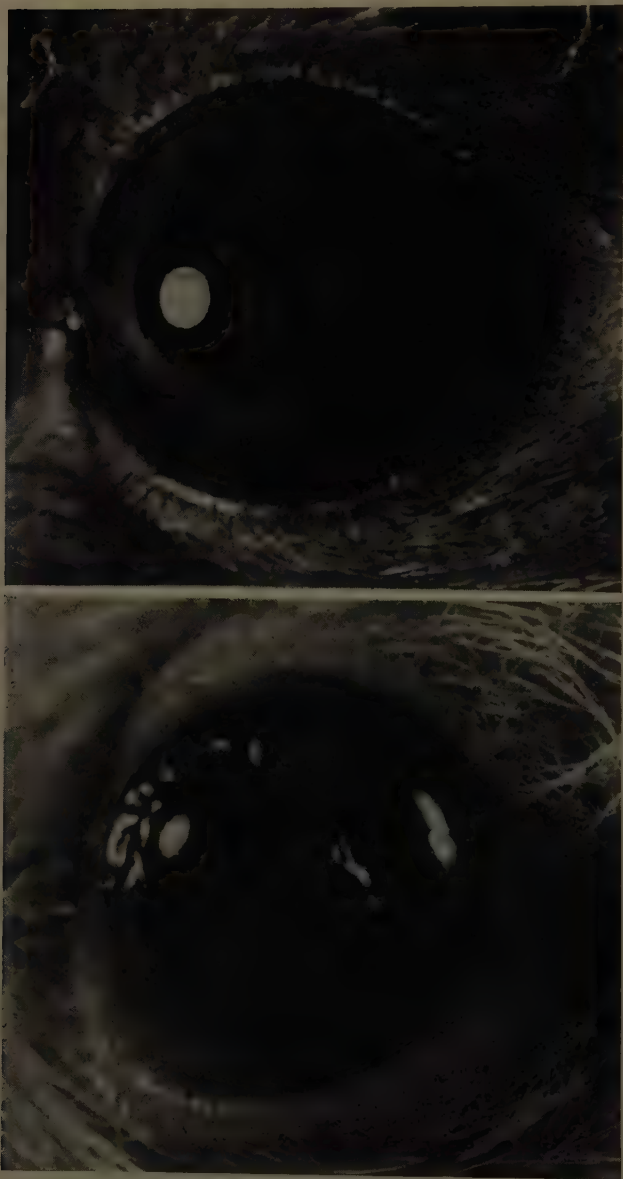


FIGURE 6. *Top*, pituitary graft growing in the anterior chamber of the eye. *Bottom*, polyethylene tube (filled with India ink for display) fixed in anterior chamber of eye; tube passes subconjunctivally, then subcutaneously, to connect with microinjector.

to maintain the TW and TRR of the PTU-treated rats at the levels of those of normal, untreated animals.

SITE(S) OF ACTION OF THYROID HORMONE IN INHIBITING TSH

The reciprocal relationship between the level of thyroidal, gonadal, and adrenocortical hormones and their respective pituitary hormones has been established. The relationship has been described in terms of "feedback" or "servomechanism" control. One step in this control system, namely, the primary site or sites of action of the target hormones, has been investigated by five different methods. An analysis of these methods and of the results

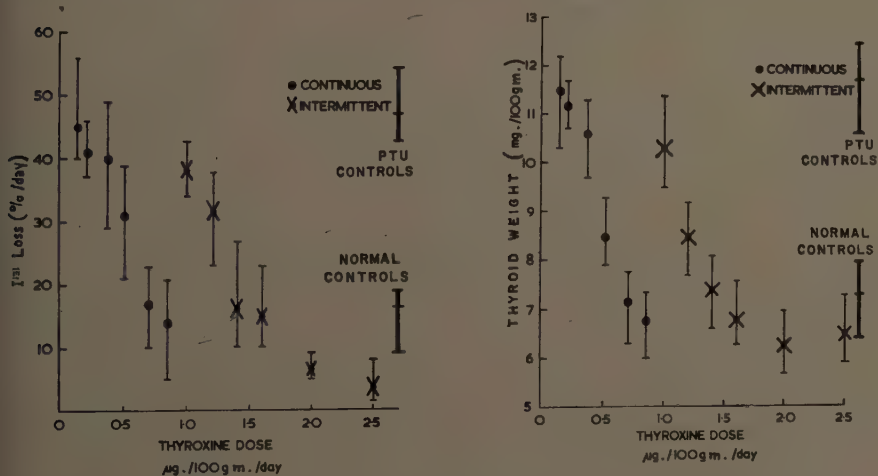


FIGURE 7. *Left*, effect of various doses of L-thyroxine on I^{131} release rates from thyroids during the 10 days the rats were receiving PTU. *Vertical lines*: each point on the scatter diagram represents the mean loss over the 10 days for a group of 6 animals. The thyroxine was given subcutaneously either as a single injection each day or by continuous infusion. *Right*, effect of various doses of L-thyroxine on the thyroid weight of rats receiving PTU for 10 days. The thyroxine was given subcutaneously either as a single injection each day or by continuous infusion. The thyroid weights (mean and maximum deviation from mean) of normal controls and PTU-treated control rats are shown by the vertical lines.

thus obtained shows that for each experiment more than one interpretation is possible; therefore, a definitive answer to this apparently simple problem is not yet possible.

Microinjection of Thyroxine into Pituitary and Hypothalamus

In a previous paper we have shown how small and systemically ineffective doses of hydrocortisone infused continuously into the pituitary fossa for 10 days inhibit the increased release of adrenocorticotrophic hormone (ACTH) that results from unilateral adrenalectomy.⁴⁹ Similarly, small and systemically ineffective doses of estradiol infused continuously and directly into the pituitary fossa cause the regression of castrate gonadotrophs. Moreover, on histological examination of these injected pituitaries a graded response from the site of injection often could be seen.⁵⁰ The control infusions of similar ineffective

amounts of hormone were given subcutaneously or just outside the pituitary fossa in the subarachnoid space in various spatial relationships to the hypothalamus. These results strongly suggest that hydrocortisone and estradiol act directly on the pars distalis, but do not prove that the pars distalis is the only area sensitive to the inhibitory effect of hydrocortisone or estradiol.

We have repeated this type of experiment with thyroxine, using TW and TRR as indices of TSH release in PTU-treated rats over a 10-day period. Various doses of L-thyroxine were given by continuous infusion in 0.1 ml. to

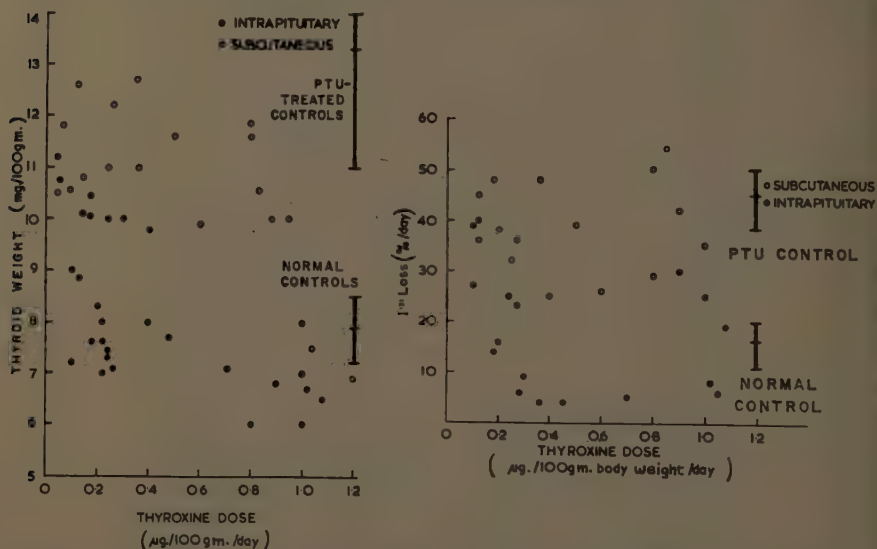


FIGURE 8. *Left*, effect of various doses of L-thyroxine on the thyroid weight of rats receiving PTU for 10 days. The thyroxine was given by continuous infusion either subcutaneously or directly into the pituitary fossa. The thyroid weights (mean and maximum deviation from mean) of normal controls and PTU-treated control rats are shown by the vertical lines. *Right*, effect of various doses of L-thyroxine on the I^{131} release rates from thyroids during the 10 days the rats were receiving PTU. Each point on the scatter diagram represents the average loss over the 10 days for an individual animal. The thyroxine was given by continuous infusion either subcutaneously or directly into the pituitary fossa. The I^{131} release rates (mean and maximum deviation from mean) of normal controls and PTU-treated control rats are shown by the vertical lines.

0.2 ml. of M/250 glycine buffer at pH 9.5 directly into the pituitary fossa or subcutaneously. For more rigid control, animals receiving thyroxine subcutaneously were operated on in the same way as was the intrapituitary group, and a control polyethylene tube was left buried in the pituitary fossa. The results are shown in FIGURE 8. The thyroïdal responses to intrapituitary and to subcutaneous injections of thyroxine in the low and high dose ranges show some overlapping. However, in the middle dose range it is clear that a continuous intrapituitary infusion of thyroxine was more effective in preventing goiter and depressing the TRR than a similar continuous subcutaneous dose. Under the conditions of this experiment, 0.2 to 0.25 μg. of L-thyroxine/100 gm./day given by continuous injection into the pituitary maintains the TW and

TRR of the PTU-treated rats at levels similar to those of the normal untreated animals. The results suggest that thyroxine acts directly at the pituitary but, again, they do not prove that this is the only area sensitive to thyroxine with regard to TSH release. The results also show that the inhibiting effect of thyroxine administered directly into the pituitary can be maintained over a 10-day period, and they suggest that here is a normal continuous physiological mechanism regulating the level of thyroid hormone in the blood.

Von Euler and Holmgren¹³ studied the effects of systemically ineffective doses of thyroxine given in single injections directly into the pituitary, median eminence, or other areas of the hypothalamus. The thyroxine was injected in volumes of 0.002 ml. to 0.005 ml., but the time taken to administer the volume was not stated. Such information would be important in determining the spread of the injected solution. These investigators concluded that the inhibitory effect of thyroxine was produced only by a direct action on the anterior pituitary.

Recent experiments of Yamada and Greer⁶⁰ and of Yamada⁵⁹ also utilized single or repeated single microinjections (0.02 ml.) of systemically ineffective doses of thyroxine into the pituitary or hypothalamus. Their results suggest that thyroxine can act to inhibit TSH release at either the pituitary or the "thyrotropin" area of the hypothalamus. After injections into the hypothalamus there was a period of 6 to 9 hours before the TSH secretion was inhibited, whereas the inhibition was immediate with injections made directly into the pituitary.

Several technical considerations are important in the interpretation of all the results obtained with microinjections. A variable mechanical spread of the injected drug occurs; this is particularly relevant when doses as large as 0.02 ml. are given in single injections^{59,60} into the rat pituitary or brain. In the latter tissue almost no extracellular fluid space can be detected by electron microscopy.^{40,52} The time taken to deliver this volume (0.02 ml.) was not published by Yamada and Greer. It should be noted that we inject 5 to 10 times this volume as a continuous infusion over 24 hours. Since drugs injected into the anterior hypothalamus can diffuse into the portal circulation and be carried down to the entire pars distalis, it is conceivable that a drug acting only at the pituitary is more effective when injected into the anterior hypothalamus than when injected directly into the pituitary; from the latter site the drug might have a variable and less efficient spread through the gland. This argument may also be applied to the experiments of Flerko and Szentagothai,¹⁹ who reported an inhibition of gonadotropic secretion when tiny bits of ovary were transplanted into the hypothalamus but not when transplanted into the pituitary. The further possibility should not be overlooked, that drugs (0.1 ml. to 0.2 ml. per day) injected slowly and continuously, as in our method, will diffuse out of the pituitary fossa up to the hypothalamus.

The cautious summary of results of experimental microinjections of thyroxine into the pituitary or hypothalamus is that the exact site or sites of the primary effect of thyroxine in inhibiting TSH release are not yet established. Moreover, they will not be determined easily until such local microinjections are made into the isolated pituitary and hypothalamus.

Effect of Thyroxine on TSH Release from Pituitary Grafts

The experiments consist essentially of grafting the pituitary in a place away from the hypothalamus. They demonstrate that such a graft releases TSH, although in reduced quantities, and that thyroxine administered systemically can reduce still further the TSH output of the grafted pituitary.^{14,28,54} These results usually are interpreted as proving a direct action of thyroxine on the pituitary. However, an alternative and equally plausible explanation is that the thyroxine acts to inhibit the release of a humoral mediator (from, for example, the hypothalamus) that reaches the grafted pituitary by the systemic circulation.

We are attempting at present to modify our experiments so that a more definitive interpretation may be made. One method, previously discussed, is to administer systemically ineffective doses directly into the pituitary graft. Another approach is to determine whether the function of the grafted pituitary depends on the ability of a hypothalamic drive to reach it via the systemic circulation. If the function of the grafted pituitary is independent of that of the hypothalamus, then the results of subcutaneously injected thyroxine would clearly prove a direct action of that hormone on the pituitary.

The Effect of Thyroxine on TSH Release from the Pituitary after Stalk Section

The pituitary stalks of rabbits were cut, and precautions were taken to prevent regrowth of the portal vessels. In none of the animals was the decreased release of TSH due to systemically injected thyroxine abolished.^{32,35} Since the pituitary and hypothalamus were no longer in intimate vascular relationship, these results have been interpreted as evidence that thyroxine acts directly on the pituitary. However, the criticism applied to the question of the response of pituitary grafts to thyroxine is valid here: the thyroxine could be acting at the hypothalamus, decreasing a drive that reaches the pituitary via the systemic circulation.

The Effect of Thyroxine on TSH Release after Hypothalamic Lesion

Several groups of investigators have shown that lesions in the anterior hypothalamus can interfere selectively with TSH release. The recent papers of Greer^{26,27} and of D'Angelo and Traum¹¹ contain many pertinent references to this finding.

Even in the presence of large lesions in the hypothalamus the release of TSH from the pituitary is regulated by thyroid hormones. Thus the thyroid:serum iodide ratio increases,²⁶ and there is a discharge of TSH from the pituitary¹⁰ when such lesioned animals are fed PTU. Moreover, thyroxine and triiodothyronine still can suppress TSH secretion.¹¹

These results suggest strongly that thyroid hormone acts directly on the pituitary. However, a hypothalamic lesion, large as it may be (regarding its compatibility with life), does not entirely eliminate hypothalamic influence on the pituitary. Therefore, the results could still be interpreted in terms of thyroxine action on the pituitary or on the remaining small and functioning hypothalamus.

Localization of Labeled Thyroid Hormones in Pituitary Gland and Hypothalamus

The selective accumulation of I^{131} -labeled thyroxine and triiodothyronine by the anterior and posterior pituitary and the hypothalamus has been studied and discussed by several investigators.^{8,9,20,21,31,57} The critical value of such localization studies, in so far as they establish the site of thyroid hormone action in inhibiting TSH release, remains in doubt. Species vary as to what lobe of the pituitary and what area of the hypothalamus best concentrate thyroxine and triiodothyronine. Localization may depend on the amount of deiodinase present in the area^{21,56} and on nonspecific differences in the permeability of the relevant blood vessels, as demonstrated by a higher accumulation of intravital dyes in both lobes of the pituitary and in the tuber cinereum and hypothalamus.^{2,58} Furthermore, it has been suggested that the concentration of thyroid hormones in the posterior lobe and paraventricular nucleus is associated with the regulation of fluid balance.²⁰

IS THERE A HYPOTHALAMIC INFLUENCE ON PITUITARY FUNCTION?

By many techniques it has been established that the pars distalis is under neural control and that the hypothalamus is of particular importance in this respect. Some experiments have demonstrated that the normal neural influence is exerted only when the pituitary is closely connected to the hypothalamus by the hypophyseal portal circulation. The important question of whether there is any hypothalamic influence on a pituitary receiving systemic blood is unanswered. Experiments that have been made in attempts to clarify this question and their results are summarized in the following:

(1) Hypothalamic lesions can produce discrete abnormalities of pituitary function, such as selective impairment of TSH release.

(2) Recently Harris demonstrated an increase in TSH release from the pituitary of rabbits when the anterior portion of the median eminence was stimulated by electric impulses. It is important to note that the "thyrotrophin" area, as determined by stimulation, agrees with lesions.^{33,36}

(3) Interruption of the intimate vascular hypothalamic-pituitary relationship by stalk section or by grafting the pituitary away from the hypothalamus causes a very marked reduction in the secretion of pituitary hormone.

Ischemia and infarction of the pituitary following the above procedures contribute in part to the loss of function. However, Harris and Jacobsohn showed that in freshly hypophysectomized rats the grafting of the pituitary under the median eminence (but not elsewhere) allowed an almost normal return of pituitary function.³⁴ Their results have been confirmed and the experiments extended in a most elegant fashion by Nikitovitch-Winer and Everett,^{41,43} who transplanted the pars distalis to the renal capsule for one month and then retransplanted it to the median eminence. After the second transplantation the female rats showed sexual cycling, became pregnant on mating, delivered small litters, and showed partial return of thyroid and adrenocortical activity. Histological examination of the grafts under the median eminence revealed a relatively normal pituitary cell structure that is never found in pituitaries grafted elsewhere.⁴⁴

These experiments leave no doubt that the hypothalamus has a special influence on the pituitary that can be exerted when the two are in close contact, and that the loss of function when the pituitary is grafted apart from the hypothalamus is not due entirely to ischemia.

It is necessary to qualify this discussion of the function of pituitary grafts by the following comments:

The pituitary grafted away from the hypothalamus can apparently maintain a normal or even higher secretion of lutotropin hormone.^{15,16,42} The suggestion has been made that the hypothalamus normally exerts an inhibitory effect on the release of this hormone and that the graft receives less of this inhibitory influence.

The determination of whether any hormone secretion is completely lost and the estimate of the relative decrease in the secretory rate of each of the hormones from the grafted pituitary depend in part on the sensitivity of the assay system for each hormone. This is illustrated by using a simple and sensitive technique that demonstrates and measures the function of the grafted pituitary. Briefly, the procedure is as follows. Rats are hypophysectomized, put on a low-iodine diet, and fed PTU. Two- to 5-day-old rats serve as donors for the tissue to be grafted. Three thyroid lobes are grafted into one eye and a mixture of 3 thyroid lobes and 3 whole pituitaries is grafted into the other eye. Any inhibitory effect of the thyroid graft on the pituitary graft is prevented by the PTU and low-iodine diet. Two weeks after grafting, the administration of PTU is discontinued; 24 hours later a tracer dose of I^{131} is given and, 15 hours later, the animals are sacrificed. The I^{131} uptake of the intact thyroids and the microscopical morphology of the grafts and intact thyroid are determined. Hypophysectomized rats without grafts but on the same PTU and low-iodine diet serve as controls.

The effect of the grafted pituitary on the intact thyroids agreed with the results of Scow and Greer.⁵³ Compared to those of hypophysectomized controls, the thyroid weights were the same; there was a slight hypertrophy of the thyroid cells and a tenfold increase in I^{131} uptake. The hypertrophy of the thyroid cells was much more marked in the thyroid graft in contact with the pituitary than in the thyroid graft alone (FIGURE 9). The pituitary-thyroid grafts weighed 5.3 ± 1.3 mg., and the thyroid grafts alone weighed 1.7 ± 1.0 mg. FIGURE 10 shows a typical section of the pituitary thyroid graft. The pituitary contributes a small fraction (estimated roughly as less than 10 per cent) to the weight of the double graft; therefore, the thyroid grafts in contact with the pituitary are several times larger than the thyroid grafts alone. These results (in contrast with those of Greer) suggest that the grafted pituitary is capable of releasing a "growth" factor as well as a "metabolic" factor. Evidence of the release of the growth factor apparently is afforded only by the more sensitive double-graft technique.

Recently some very surprising results have been published concerning the function of pituitaries grafted apart from the hypothalamus. Goldberg and Knobil²⁴ observed that after several months some male rats with transplanted pituitaries showed a return of sexual function. The testes descended into the scrotums, the animals were able to sire litters, and histological examination of their testes showed fully developed germinal epithelium and mature sperma-

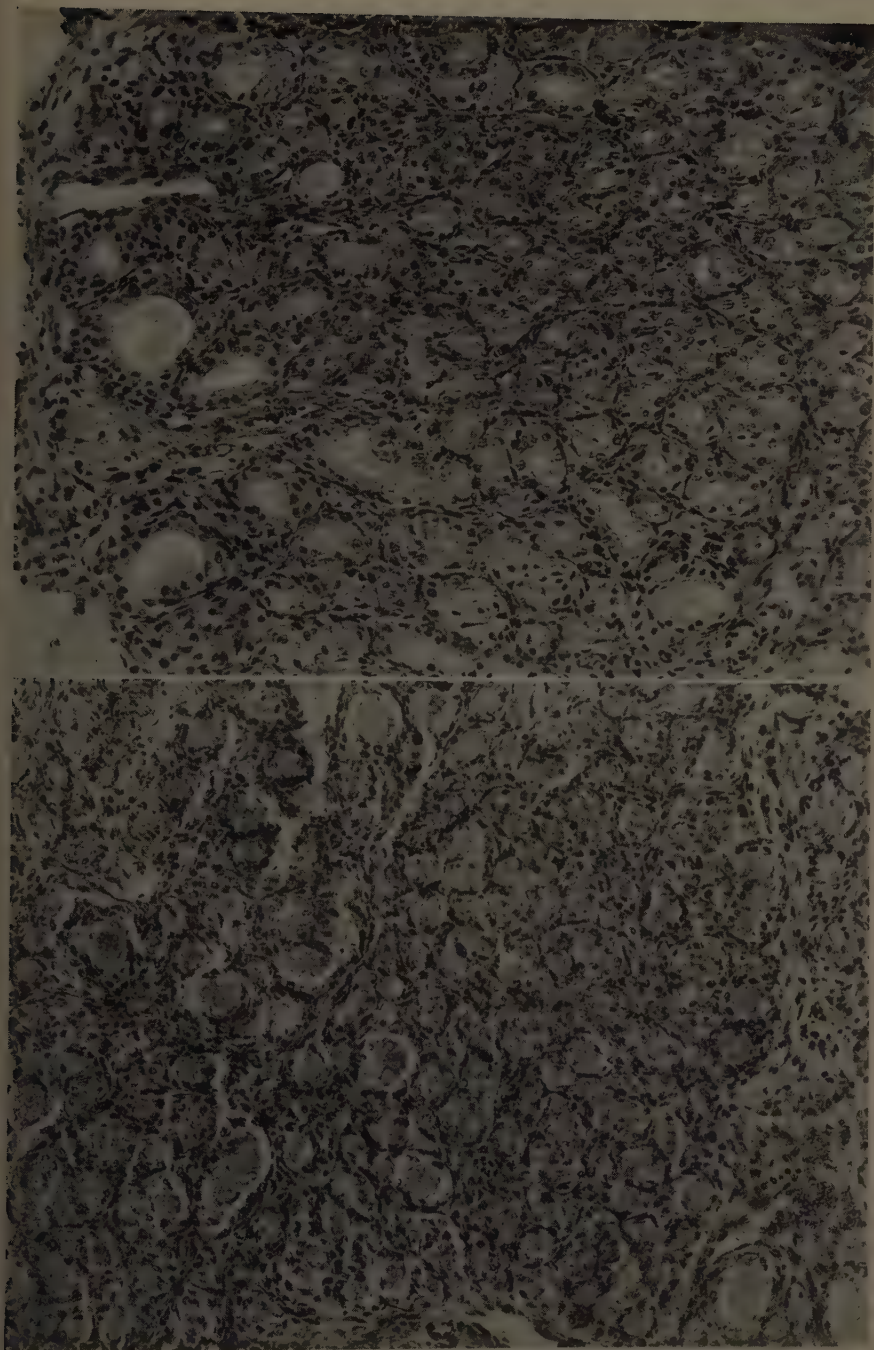


FIGURE 9. *Top*, section from hypophysectomized rat on PTU and low-iodide diet. Thyroid graft from the double graft (thyroid graft in contact with and growing in the same eye or pituitary graft). Note marked hypertrophy of thyroid epithelial cells. $\times 205$. *Bottom*, section from hypophysectomized rat on PTU and low-iodide diet. Thyroid graft alone. The other eye carried the double graft. Note only slight hypertrophy of thyroid epithelial cells. $\times 205$.

tozoa. Similar results in such long-term experiments have been reported by Long,³⁹ who describes some body growth and partial return of thyroid and adrenal function. Long emphasizes that the return of these functions was observed only after several months and that the return of the various pituitary functions was not uniformly evident. On the other hand, the return of gonadal function in female rats with transplanted pituitaries has not been observed. The interpretation of these results with respect to the question of direct (via the hypophyseal portal circulation) hypothalamic regulation of the anterior pituitary is uncertain.

(4) Physiological stimuli other than the alteration of thyroid hormone level in the systemic blood can alter TSH release. Thus, in the rabbit, moderate

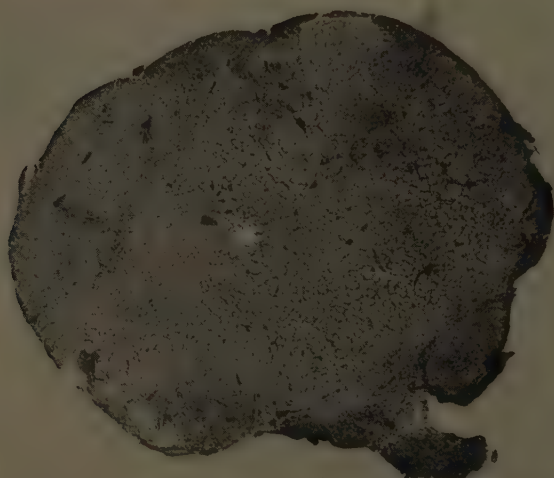


FIGURE 10. Section from hypophysectomized rat on PTU and low-iodide diet. Thyroid-pituitary graft in same eye. Note that most of the double graft is comprised of thyroid tissue. $\times 27$.

cold causes an increase in TSH release within half an hour.⁵ Because of the short latent period it is believed that a nervous reflex mediated through the hypothalamus rather than a fall in thyroid hormone level in the blood is responsible. A similar argument is applied to the decreased TSH release caused by emotional and physical stress.^{4,6,55} However, since cortisone can inhibit TSH release in the rabbit⁴ the decreased TSH release consequent on stress may not represent a primary effect of the hypothalamus on pituitary TSH release. That cortisone and physical stress still appear to be effective after stalk section or transplantation of the pituitary^{7,14} is compatible with this view.

(5) Neuropharmacological studies relating to pituitary physiology have been of limited value. This is largely because of the nonspecificity of their action and the difficulty of determining their primary and other sites of action.

Nevertheless, blocking drugs can be used successfully as tools to study various aspects of pituitary physiology. Everett¹⁷ and others have shown that a

number of blocking drugs can prevent ovulation in the rat. The use of these drugs has not much advanced our knowledge of the sequence and chemical nature of the links in the brain-hypothalamic-pituitary regulation system for ovulation. The main value of the studies has been to demonstrate that there exists in rats during the day of proestrus a critical period, when the pituitary is being stimulated to release ovulating hormone.^{1,18} It has been shown further that the release of an adequate amount of ovulating hormone from the rat pituitary takes about half an hour.^{15,16}

We have been studying the effect of atropine on TSH release. The main purpose of our experiments was to determine whether atropine may be used as a tool to block neural mechanisms concerned with TSH release in the hypothalamus, reticular formation, or elsewhere. If this were possible, atropine could be applied locally and in systemically ineffective doses to the neural tissue. Any effect on the TSH release from the grafted pituitary would suggest strongly that the hypothalamus was producing a humoral substance able

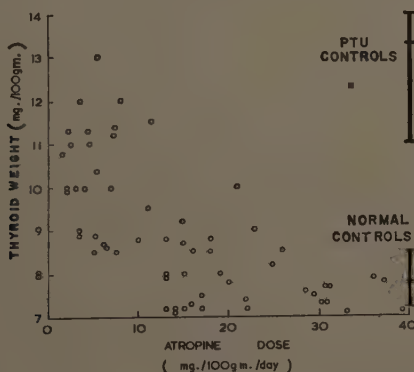


FIGURE 11. Effect of various doses of atropine sulfate on the thyroid weight of rats receiving PTU for 10 days. The atropine was given by continuous subcutaneous infusion.

to reach the pituitary by the systemic circulation and thereby to influence its TSH release.

We fed rats 20 mg. PTU daily for 10 days, and determined the effect of various doses of atropine sulfate on the TW and TRR. The results given in FIGURES 10 and 11 demonstrate that atropine by continuous subcutaneous infusion can block TSH release for at least a 10-day period. These experiments were repeated, the atropine being given by continuous infusion directly into the pituitary fossa or just outside the fossa under the hypothalamus. The results, shown in FIGURES 12 and 13, demonstrate that atropine at one tenth of the systemic dose level, infused into the pituitary fossa or under the hypothalamus, can inhibit TSH release over a 10-day period.

At the present time we are not able to say with certainty whether the atropine was diffusing from one site to another, or whether the pituitary and neural tissue are both sensitive to atropine in causing inhibition of TSH release. Moreover, the problem will not be solved in a definitive manner until local micro-injections are given to the isolated pituitary or hypothalamus. Such experiments are now in progress.

MECHANISM(S) BY WHICH HYPOTHALAMUS REGULATES TSH RELEASE FROM PITUITARY

In the previous section evidence has been presented to show that the nervous system and, in particular, the hypothalamus regulate the pars distalis.

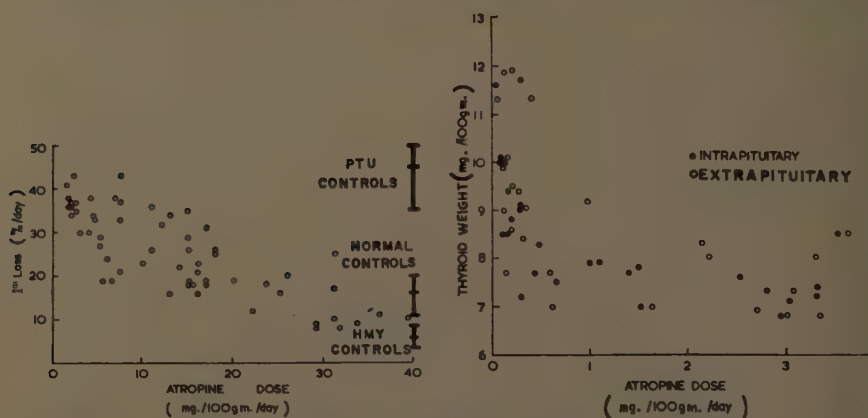


FIGURE 12. *Left*, effect of various doses of atropine sulfate on the I^{131} release rates from thyroids of rats receiving PTU for 10 days. Each point on the scatter diagram represents the average loss over the 10 days for one animal. The atropine was given by continuous subcutaneous infusion. *Right*, effect of various doses of atropine sulfate on the thyroid weights of rats receiving PTU for 10 days. Atropine given by continuous infusion directly into the pituitary fossa or just outside the pituitary fossa. See text.

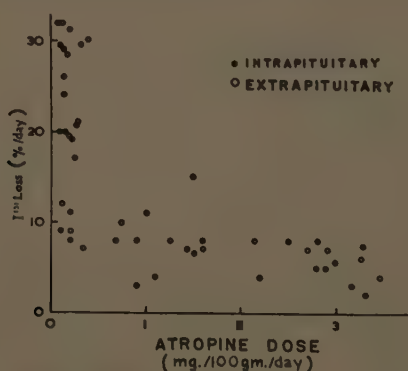


FIGURE 13. Effect of various doses of atropine sulfate on the I^{131} release rates during the 10 days the rats were receiving PTU. Each point on the scatter diagram represents the average loss over the 10 days for one animal. The atropine was given by continuous infusion directly into the pituitary fossa or just outside it. See text.

Hypothalamic stimulation³⁶ and exposure to cold⁵ cause an increase in TSH release, suggesting that the hypothalamus is capable of regulating the pars distalis and altering the thyroid hormone level in the blood. An alternative method of expressing this relationship is to state that the hypothalamus can stimulate or increase TSH release in the presence of a high systemic thyroid hormone level. This is supported by the observation that 50 or 100 μ g. thy-

roxine administered to a rabbit just before the exposure to cold will not prevent the increased blood concentration of TSH.³ Similarly, it may be impossible to inhibit ACTH secretion in animals under severe stress by the administration of adrenocorticosteroids.²³ Whether the hypothalamus under other circumstances also can act in such a way as to maintain a constant thyroid hormone level in the blood is not known.

The assumption is usually made that the same principles apply to the regulation of each of the pituitary hormones. Thus, if estrogen and hydrocortisone act directly on the pars distalis^{50,51} it could be predicted that this would also be the site of action of thyroxine: a conjecture supported by the results presented in this paper. Thus, in discussions of possible mechanisms of TSH release, data and notions derived from experiments dealing with the regulation of other pituitary hormones (as in the review by Sayers *et al.* concerning ACTH⁵¹) may be considered.

(1) The most attractive theory is that the median eminence elaborates a specific neurohumoral mediator that is carried via the portal circulation to the pars distalis. The chemical, in lower but effective concentration, also may be able to reach the grafted pituitary through the systemic circulation. If interference with the hypothalamus (lesion, stimulation, or local neuropharmacological block) altered the function of the grafted pituitary, this would constitute stronger evidence for the existence of a neurohumoral agent than would the same procedures carried out with the pituitary *in situ*. Moreover, it would be reassuring to have data suggesting that a chemical indeed existed, before attempting the difficult task of isolating and testing hypothalamic or hypophyseal portal blood extracts. An adequate test system (simulating normal events) may require the extract to be infused directly into the pituitary over a certain period of time. The definitive experiment also may require that the animal have a hypothalamic lesion in order that the endogenous source of the proposed chemical be eliminated.

The possibility has been suggested that the neurosecretory material associated with the hypothalamic-neurohypophyseal complex is liberated into the hypophyseal portal vessels in order to regulate the release of TSH.^{12,22,54} However, the observations of others do not support the suggestion.^{45,46} We could find no evidence for an increased TSH release from the grafted or intact pituitary perfused directly and continuously with various concentrations of posterior pituitary extracts, acetylcholine, serotonin, or epinephrine (unpublished data).

The hypothalamus also may elaborate an inhibitory neurohumoral chemical, as suggested by two sets of observations. The grafted pituitary (presumably receiving a decreased concentration of the neurohumoral chemical) continues to secrete normal or even increased amounts of luteotrophin (LTH).^{15,16,42} This seems to suggest that the increased LTH secretion that results from the stimulus of suckling or from sterile coitus is due to a decreased inhibition of LTH release. Pituitary grafts in rabbits secrete more TSH than do normal pituitaries in stressed animals,^{6,14} a fact suggesting that in the latter animals the release of TSH is being inhibited.

(2) Brown-Grant recently has suggested a method by which the hypothalamus may regulate TSH release.⁴ The hypothalamus is envisaged as removing

a fraction (capable of regulation) of the thyroid hormone from the arterial blood supplying the primary plexus of the hypophyseal portal system, thus exposing the pituitary to a modified and regulated thyroid hormone content. The vascular anatomy of the region appears suitable to such a mechanism, and the theory is certainly compatible with many experimental observations, although there are no definitive data to support it. Moreover, if this were the only mechanism of hypothalamic influence, it would be difficult to explain the difference between the TSH release of the PTU-treated normal animal and that of the PTU-treated hypothalamic-lesioned animal. In the latter there is virtually no thyroid hormone in the blood, and therefore a "filter system" could have very little influence.

(3) Other miscellaneous theories will be listed briefly. That alternative opinions exist is an indication of the general lack of knowledge. Neurohumoral agents may be released from the much-disputed nerve endings in the adrenohypophysis.

Several experiments have suggested that the release of ACTH is regulated by the level of ACTH in the blood. This regulation is an extra-adrenal action of ACTH; however, it is not known whether the site of action is the pituitary, hypothalamus, or elsewhere.^{37,38} A similar regulation may apply to TSH, but has not been tested.

The portal vessels may have special physiological characteristics regarding permeability and also may be able to regulate the blood flow to the pituitary.

The portal vessels per se may not be critical; instead, a specific connective tissue from the median eminence may grow down with the vessels and have a specific trophic influence on the epithelial elements of the adenohypophysis. This would be analogous to the specific relation existing between metanephrogenic mesenchyme and induction of kidney tubules.^{29,30} If such a trophic relationship exists for the pituitary, its demonstration and the isolation of the chemical involved will be extremely difficult.

SUMMARY

A technique of prolonged continuous microinjection of chemicals in the unanesthetized unrestrained rat is described. The chemicals can be delivered subcutaneously, directly to the pituitary *in situ*, to the grafted pituitary in the anterior chamber of the eye, and under or in the hypothalamus. The advantages and some of the results of the technique are described.

Difficulties in the design and interpretation of experiments studying regulation of TSH release are discussed. It is emphasized that many of the conclusions concerning various aspects of TSH release are based on suggestive rather than on definitive data.

Three main problems concerning TSH release have been discussed.

(1) Site(s) of action of thyroid hormone in inhibiting TSH release. Five different techniques have been employed. The evidence, though not definitive, strongly favors a direct effect of thyroxine on the pars distalis. The possibility that thyroxine acts at both the hypothalamus and pituitary cannot be overlooked.

(2) Is there a hypothalamic influence on the pituitary? It seems certain that the central nervous system and, particularly, the hypothalamus exert an

important influence on the pars distalis. This influence has been demonstrated by the effect, on TSH release, of hypothalamic lesions and stimulation, of grafting the pituitary under the median eminence or away from the hypothalamus, of physiological stimuli and of neuropharmacological blocking drugs.

(3) Mechanisms by which the hypothalamus may regulate TSH release. The hypothalamus exerts its full influence on the pituitary only when these two structures are in close proximity and in intimate vascular relationship by way of the hypophyseal portal circulation. It is not known whether the hypothalamus can exert any influence on the grafted pituitary via the systemic circulation.

The hypothalamus is able to alter the secretion of TSH by mechanisms other than the alteration of the thyroid hormone level in the systemic blood. A constant thyroid hormone level in the blood is maintained partly by the pituitary-thyroid axis. Whether the hypothalamus also acts in this way is not known.

At the present time the most attractive theory is that the hypothalamus secretes humoral mediators (activating and possibly inhibiting) into the hypophyseal portal circulation, and that the pituitary responds to these mediators and to the thyroid hormone level in the blood.

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THE PARTICIPATION OF THE NERVOUS SYSTEM IN THE CONTROL OF THYROID FUNCTION*

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There is now little question that the central nervous system plays an important role in the regulation of thyroid function. Several laboratories have reported observations indicating that the integrity of the hypothalamohypophyseal system is necessary for the maintenance of normal thyroid function. Most of these studies recently have been summarized^{1,2} and will not be reviewed here. Rather, we shall outline briefly our concept of the current status of the problem and summarize recent experiments in our laboratory.

Central Nervous System Lesion

There is general agreement that destruction of a portion of the anterior hypothalamus decreases thyroid function. The precise location of this "thyrotropin area" is in mild dispute, but most investigators report that it lies in or near the mid-line between the optic chiasm and the median eminence. Our own studies, which have been confined primarily to the rat, indicate that its anterior limit is the paraventricular nucleus and that it lies above the base of the hypothalamus.³ Destruction of this area inhibits the goitrogenic response to antithyroid drugs and decreases both radioiodine uptake and thyroïdal secretion rate. Since animals with anterior hypothalamic lesions will respond normally to exogenous thyrotropin (TSH),⁴ it has been assumed that these effects are due to a decreased secretion of hypophyseal thyrotropin.

As yet, destruction of other areas of the brain has not been found to interfere significantly with thyroïdal function. Mess⁵ has reported that electric ablation of the habenular nuclei reduces TSH secretion, but studies in our laboratory have not confirmed this.⁶ Similarly, we have found that bilateral amygdalectomy,⁷ decortication,⁸ and destruction of the pineal and subcommissural organ⁶ do not alter thyroid function significantly.

Pituitary Transplants

Separation of the pituitary from its normal hypothalamic connections either by stalk section or by transplantation generally has been found to result in a striking decrease of its secretory potency.⁹ TSH secretion, however, is maintained to a considerable extent.² Although thyroid weight declines to the level seen after hypophysectomy, radioiodine uptake and thyroïdal iodide concentration, as measured by the thyroid/serum iodide ratio, remain at an approximately normal level. Histologically, the thyroid appears to be between the normal and hypophysectomized states.

TSH secretion still can be altered by changes in the level of circulating thyroid hormone in animals with transplanted pituitaries. Lowering the amount

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of circulating thyroxine by chronic administration of propylthiouracil increases greatly both radioiodine uptake and the thyroid/serum iodide ratio,¹⁰ while administration of thyroxine causes a decline in both.^{10,11} In spite of the marked changes in iodine metabolism thus produced, very little change is produced in thyroid weight, although the histological appearance of the gland is obviously altered. Thus some regulating mechanism still can act on the transplanted pituitary to maintain a stable level of circulating thyroxine.

Stimulation of the Nervous System

Harris and Woods¹² have demonstrated that electric stimulation of the anterior hypothalamus in rabbits causes a pronounced increase in thyroïdal secretion. Our efforts to produce a similar effect in the rat thus far have been unsuccessful. It is not known whether our failure has been due to technical difficulties or to a species difference.

Thyroid function also may be controlled, although very slightly, by the cervical sympathetic nerves.¹³ Following electric stimulation of the cervical sympathetic nerve in the dog, an increase of the protein-bound iodine¹³¹ concentration and output in the thyroïdal venous blood is observed. Since such an increase occurs even in dogs with transplanted pituitaries, it appears that this nerve can modify directly the hormonal secretion of the thyroid gland and does not necessarily first produce increased TSH release from the pituitary.

Site of Action of Thyroxine in Regulating TSH Secretion

It has been suggested that thyroid hormone controls TSH secretion by altering the activity of the hypothalamus and thereby that of the pituitary. Such a hypothesis is appealing, but adequate explanation must be made for the fact that isolated pituitaries can adjust their TSH secretion to the level of circulating thyroxine. Von Euler and Holmgren¹⁴ made microinjections of systemically ineffective amounts of thyroxine into the anterior pituitary and hypothalamus of rabbits and found that an inhibition of thyroïdal secretion was produced only by the intrahypophyseal injection. They concluded that thyroxine regulated TSH secretion solely by acting directly on the adenohypophysis.

Since the hypothalamus obviously plays an important role of some sort in controlling thyroid function, it was considered of interest to reinvestigate the problem. Accordingly, microinjections of systemically ineffective amounts of thyroxine were made into the pituitary and various areas of the hypothalamus of the rat,¹⁵ and the effect on thyroïdal secretion was studied. In contrast to the results of von Euler and Holmgren, it was found that thyroxine introduced either into the anterior hypothalamic thyrotropin area or into the adenohypophysis caused a significant reduction in thyroïdal secretion (FIGURES 1 and 2). Injection of thyroxine into the preoptic or posterior hypothalamic areas or into the subarachnoid space was without effect. The inhibition produced by intrahypophyseal injection occurred very rapidly, whereas there was a delay of 6 to 9 hours after intrahypothalamic injection. In both cases the duration of inhibition was approximately 20 hours. Injection of systemically ineffective amounts of thyroxine into the anterior hypothalamic area also prevented the goitrogenesis usually produced by chronic administration of propylthiouracil.¹⁶

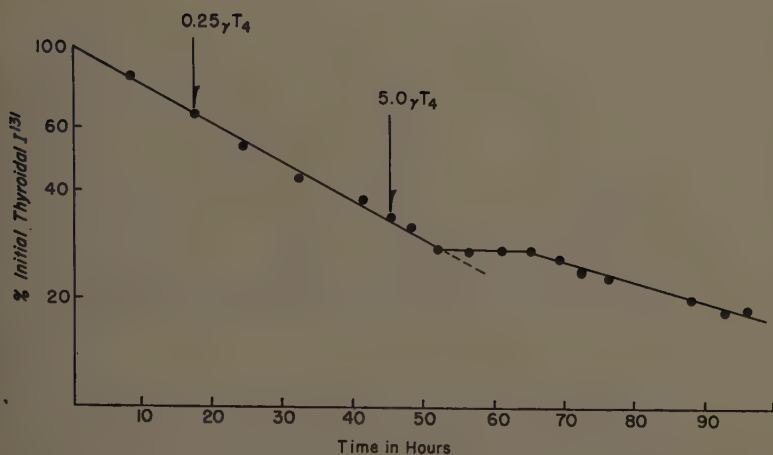


FIGURE 1. Effect of different systemic doses of thyroxine on thyroidal secretion rate in the rat. Twenty μc . I^{131} given 24 hours before initial count, and 30 mg. propylthiouracil daily, subcutaneously, started at that time. *In vivo* counts over the thyroid region made every few hours. The decline in thyroidal radioactivity is an indication of the thyroidal secretion rate. It may be seen that 0.25 μg . thyroxine (T_4) had no effect on thyroidal secretion, whereas 5.0 μg . produced a definite, though transient, inhibition. (Reproduced by permission of *Endocrinology*.¹⁶)

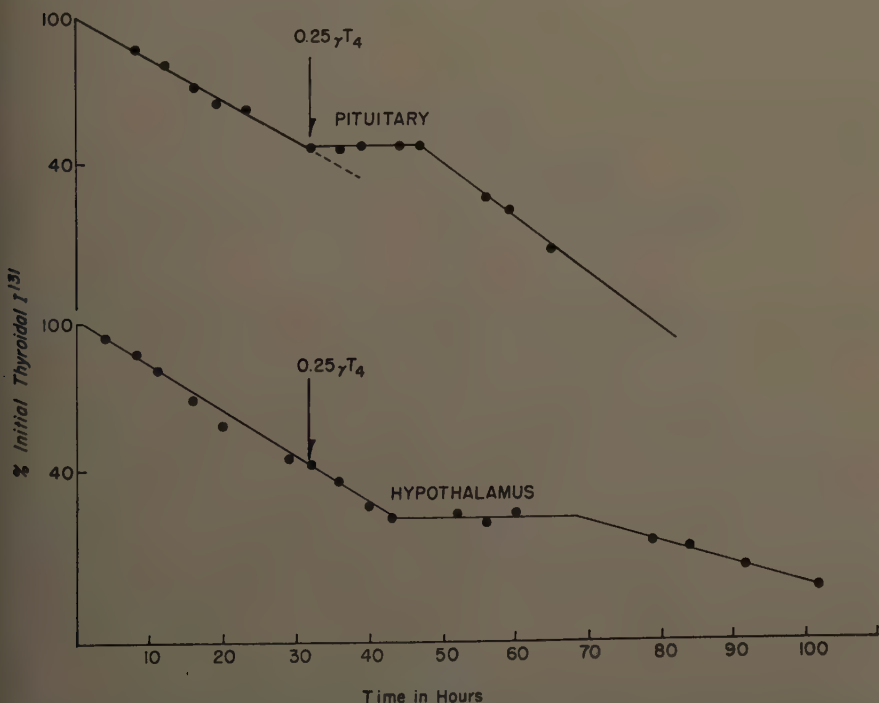


FIGURE 2. Effect of systemically ineffective amounts of thyroxine (T_4) on thyroidal secretion when injected directly into the hypothalamus or pituitary. Technique as in FIGURE 1. It may be seen that transient inhibition was produced by injection into both areas, but that the effect was considerably delayed following intrahypothalamic injection. (Reproduced by permission of *Endocrinology*.)

Since it was possible that the thyroxine introduced into the hypothalamus had been transported through the hypophyseal portal system to the adenohypophysis, there to exert its effect, the sensitivities of the hypothalamic thyrotropin area and of the pituitary to graded doses of thyroxine were compared.¹⁷ It was found that the minimal dose necessary to inhibit thyroïdal secretion was approximately the same for both areas (FIGURE 3). Since less than 1 per cent of radioactive thyroxine introduced into the hypothalamus reached the anterior pituitary, it appeared that two separate areas could act as "thermostats" in regulating TSH secretion in response to local alterations in the concentration of thyroxine.

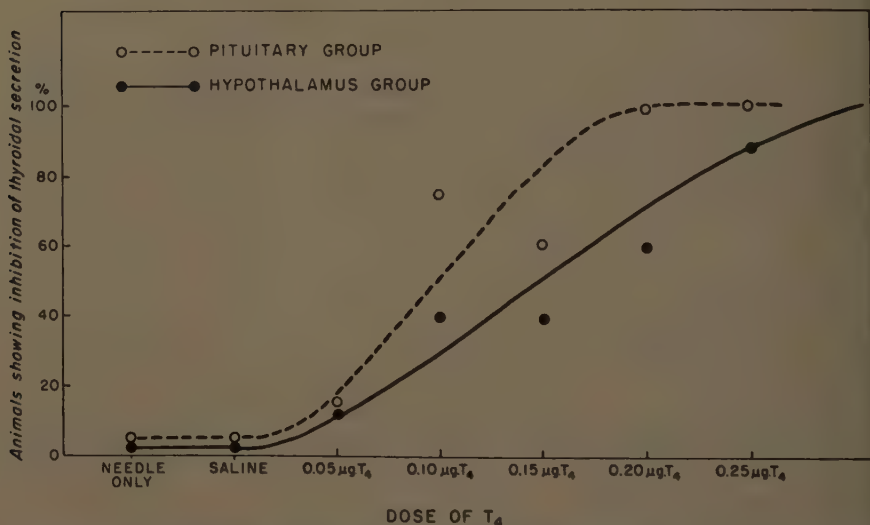


FIGURE 3. Comparison of sensitivity of the hypothalamus and pituitary to local changes of thyroid hormone concentration. (Reproduced by permission of *Endocrinology*¹⁷.)

One criticism that may be attached to these studies is that the volume injected (0.02 ml.) was quite large in relation to the size of the rat hypothalamus and pituitary. Therefore some diffusion into contiguous areas, including the third ventricle, undoubtedly occurred. Even so, the concentration in surrounding areas must have been insufficient to produce an inhibitory effect, since preoptic and posterior hypothalamic injections into areas very close to the thyrotropin center were generally ineffective. Nevertheless, more precise localization will require the utilization of smaller volumes. The minimal effective intrahypothalamic or intrahypophyseal dose also is much larger than would be expected if an equal distribution of the minimal effective systemic dose were assumed. This again is probably related to the rapid diffusion of locally injected thyroxine from the actual receptor area due to the comparatively large volume of fluid utilized.

*Effect of Intrahypothalamic or Intrahypophyseal Injections of Substances
Other Than Thyroxine on Thyroid Function*

Although current opinion favors the existence of "neurohumors" that pass from the median eminence of the hypothalamus through the hypophyseal

TABLE 1
EFFECT OF INTRAHYPOTHALAMIC OR INTRAPITUITARY INJECTION
OF CERTAIN MATERIALS ON THYROIDAL I¹³¹ RELEASE*

Materials and dose	Hypothalamus				Pituitary			
	+	±	-	0	+	±	-	0
Acetylcholine (μg.)								
1 × 1				5				
1 × 2	3			3				
10 × 2				1				
50 × 2				1				
Mecholyl (μg.)								
10				4				
Norepinephrine (μg.)								
0.1 × 2				3				
1.0 × 2				3				
10.0 × 2				3				
Epinephrine (μg.)								
0.1				2				
1.0				2				
Histamine (μg.)								
0.1 × 2	1			1				
TSH (U.S.P. mU.)								
4 × 3				2				
8 × 1				1				
8 × 2				4				4
15 × 1				1				
20 × 1				4				
Potassium iodide (μg.)								
5		1	1	4	2		1	1
2,4-Dinitrophenol (μg.)								
100.0				8				7
Hypothalamus extract					3	1	3	24
Serotonin (μg.)								
1								5
Trypan blue (mg.)								
0.1								4
0.2								4
1.0							4	

* All material injected in 0.02 ml. saline. Where repeated administration is indicated, the injections were 4 hours apart. Hypothalamus extract included aqueous and acetone extracts of fresh rat hypothalamus. Symbols: + = increased rate of thyroidal I¹³¹ release following injection; ± = equivocal effect; - = decreased thyroidal I¹³¹ release; 0 = no change.

portal system to the adenohypophysis, where they stimulate or depress the secretion of specific pituitary hormones, unequivocal demonstration of their existence has yet to be made. Several substances have been suggested as possible mediators in the nervous control of the anterior pituitary. Some have been tested in our laboratory for their possible influence on thyroidal

secretion. These test substances have been injected in a volume of 0.02 ml. into either the hypothalamus or the pituitary, and their effect on the thyroïdal release of radioiodine has been studied. The results are summarized in TABLE 1.

Acetylcholine, Mecholyl, serotonin, epinephrine, norepinephrine, histamine, and potassium iodide were all without consistent effect on the thyroïdal release of radioiodine in propylthiouracil-blocked rats (unpublished data). Occasionally an increase in the secretion rate was seen but, since this usually appeared rather late, we believe that it probably represented some mechanism unrelated to the administration of the drug. An inhibition was seen about

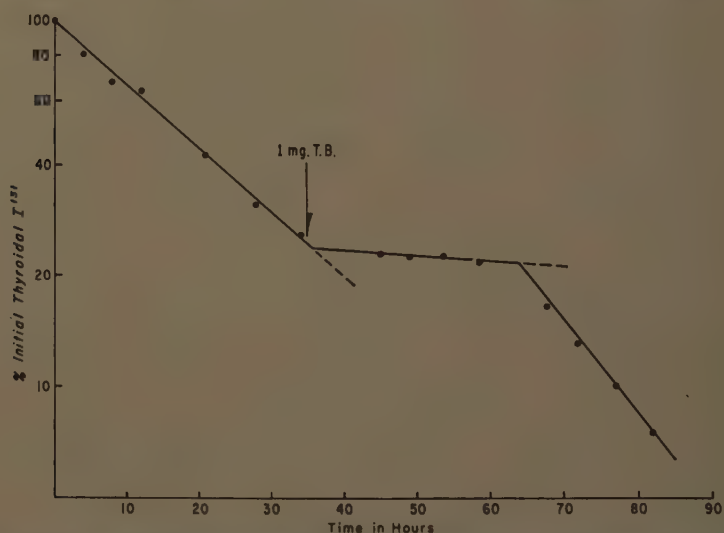


FIGURE 4. Effect of intrapituitary injection of trypan blue on thyroid hormone secretion. One tenth the minimal effective dose of trypan blue causes pronounced inhibition of thyroidal secretion when injected directly into the hypophysis. Technique as in FIGURE 1. T.B., trypan blue. (Reproduced by permission of *Endocrinology*.²⁰)

as frequently with the same material. As yet, hypothalamic homogenates or extracts injected into the adenohypophysis also have been ineffective (unpublished data).

Trypan blue has been found to produce a pronounced decrease in thyroid function when injected systemically.¹⁸ It was originally postulated that such decreased activity was related to a competition between the dye and thyroxine for binding sites on serum proteins.¹⁹ The resultant increase in free thyroxine supposedly acted on the hypothalamopituitary system to depress TSH secretion. However, it was found that one tenth of the minimal effective systemic dose produced thyroidal inhibition when injection was made directly into the pituitary²⁰ (FIGURE 4). Trypan blue apparently specifically inhibits TSH, since the adrenals and gonads seemed unaffected. It also causes the appearance of an abnormal serum protein between α -1 and α -2 globulins²¹ (FIGURE 5). Intrapituitary injection of one one-hundredth of the minimal systemic dose

necessary for the production of this serum causes it to materialize. Conversely, hypophysectomy prevents its appearance, even when large systemic doses are used.²⁰

It has been suggested that certain tropic pituitary hormones, particularly corticotropin,^{22,23} may be concentrated in the adenohypophysis and exert some direct inhibitory effect there. For this reason a study was made of the effect of local injections of from 4 to 20 mU. TSH directly into the pituitary or

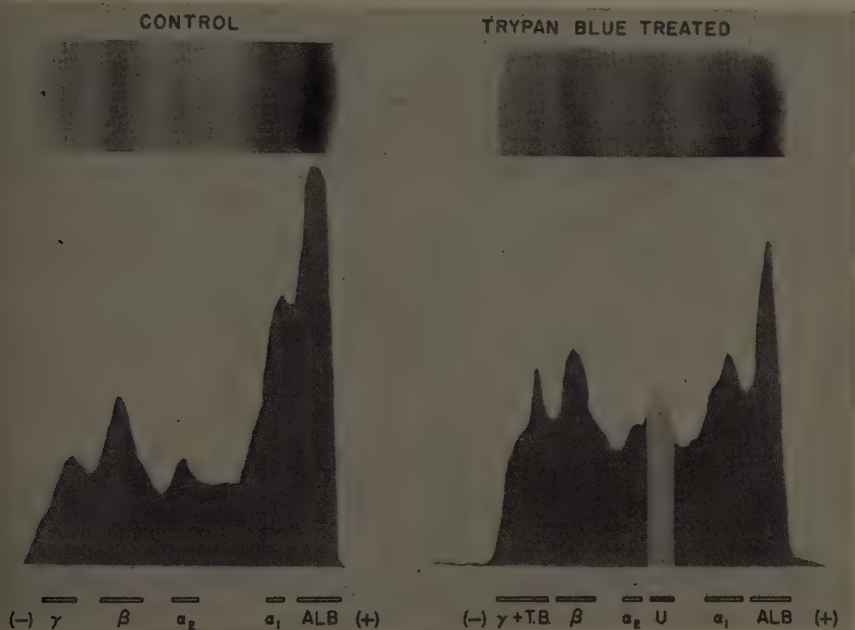


FIGURE 5. Paper electrophoresis of rat serum pH 8.6. Abnormal serum protein component (U) appearing in serum of trypan-blue treated rats. Intrapituitary injection of one-hundredth the minimal effective systemic dose causes appearance of this compound in the serum. ALB, albumin; α_1 , α_2 , β , γ , globulins. (Reproduced by permission of *Endocrinology*.²¹)

hypothalamus. No effect on thyroidal secretion was produced by injection of any dose level into either location (unpublished data).

Considerable interest has been aroused recently over the effect of dinitrophenol (DNP) on thyroid function. It has been reported that DNP causes a decrease in TSH secretion that is associated paradoxically with a fall in protein-bound iodine.²⁴ Consequently, it has been postulated that a mechanism other than the level of circulating thyroid hormone affects the servomechanism regulating TSH secretion. Although it had been suggested that the depression of TSH secretion might be due to a nonspecific increase in the metabolic rate or in body temperature due to the action of DNP, it was considered that DNP might directly inhibit either the hypothalamic or hypophyseal receptor area. However, the injection of 100 μ g. DNP into either area had no effect on thyroidal secretion (unpublished data).

Summary

Investigations involving stimulation or destruction of various parts of the central nervous system indicate that only the anterior hypothalamus is of importance in modulating thyrotropin release from the adenohypophysis. The cervical sympathetic nerves also may exert a direct although minor influence on thyroidal secretion. In spite of the importance of the anterior hypothalamus to thyrotropin secretion, the servomechanism that regulates thyrotropin release in response to the level of circulating thyroid hormone is operative in animals with heterotopic pituitaries. Microinjection of systemically ineffective amounts of thyroxine into either the anterior hypothalamus or the pituitary suppresses thyrotropin release. Sensitivity comparisons of these two regions suggest that there are two separate "receptor" areas responsive to local alterations in thyroxine concentration. The more slowly reacting center lies in the hypothalamic "thyrotropin area", whereas the more rapidly reacting receptor lies in the pituitary itself.

Trypan blue apparently directly inhibits thyrotropin secretion without decreasing the secretion of other tropic pituitary hormones. Associated with the decreased thyrotropin release, an abnormal inter- α -globulin appears in the serum. No consistent effect on TSH release was obtained with intrahypothalamic or intrahypophyseal injection of a variety of hypothetical "neurohumoral" substances or hypothalamic extracts.

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